

**Honey Proteins and their Interaction with Polyphenols**

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I dedicate this thesis to God for giving me the strength to keep going in this long journey, even when my strength seemed to give up.

To my husband and our son for being so supportive and for their encouragement all the time. To my parents and my sister for being there for me and to my extended family of friends and colleagues, who continuously encouraged me.

## Abstract.

This project aimed to determine the protein profiles and concentration in honeys, effect of storage conditions on the protein content and the interaction between proteins and polyphenols. Thirteen honeys from different botanical origins were analyzed for their protein profiles using SDS-PAGE, protein concentration and phenolic content, using the Pierce Protein Assay and Folin-Ciocalteu methods, respectively. Protein-polyphenol interactions were analyzed by a combination of the extraction of honeys with solvents of different polarities followed by LC/MS analysis of the obtained fractions. Results demonstrated a different protein content in the tested honeys, with buckwheat honey possessing the highest protein concentration. We have shown that the reduction of proteins during honey storage was caused, partially, by the protein complexation with phenolics. The LC/MS analysis of the peak eluting at retention time of 10 to 14 min demonstrated that these phenolics included flavonoids such as Pinobanksin, Pinobanksin acetate, Apigenin, Kaemferol and Myricetin and also cinnamic acid.

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## List of Abbreviations and Acronyms

Ala	Alanine
Apal	Apalbumin,
APS	Ammonium persulfate
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
Arg	Arginine
BSA	Bovine Serum Albumin
EDTA	Ethylenediaminetetraacetic acid
ESI	Electro spray ionization
EtOH	Ethanol
Gaba <sup>b</sup>	Gamma-aminobutyric
GAE	Gallic acid equivalents
GOX	Glucose-1 – oxidase
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
HGP	Hypopharyngeal gland proteins
His	Histidine
HPLC	High Performance Liquid Chromatography
Ile	Isoleucine
LC/MS	Liquid Chromatography Mass Spectrometry
Leu	Leucine
Lys	Lysine

MAP	Mitogen Activated Protein
MeOH	Methanol
Met	Methionine
MW	Molecular weight
Orn	Ornithine
Phe	Penylalanine
Pro	Proline
ProOH	Propanol
RJ	Major Royal Jelly Proteins
RT	Retention time
RT	Room temperature
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
Ser	Serine
SN	Supernatant
SPE	Solid-phase extraction
TEMED	Tetramethylethylenediamine
Thr	Threonine
TIC	Total Ion Current
TNF $\alpha$	Tumor Necrosis Factor
Trp	Tryptophan
Tyr	Tyrosine

Val	Valine
WIPK	Mitogen-activated protein

# 1.Introduction.

Honey is defined as the natural sweet substance produced by *Apis mellifera* from the nectar of plants or from secretions of living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Codex Alimentarius, 2001). Therefore some honey components originate from plant nectars and pollen, others are added by honeybees. In addition, new complexes are formed due to biochemical reactions during honey maturation, post-harvest processing and storage (Iglesias et al., 2006).

There are different varieties of honeys depending on the botanical source from which nectars were collected. They are grouped in two main types of honeys: blossom or nectar honeys and honeydew honeys obtained from excretions of plant-sucking insects on the living part of plants or secretions of living parts of plants (Codex Alimentarius, 2001). Most of the summer honeys are blended honeys because they are harvested by bees from several neighboring plants (Iglesias et al., 2006).

Honey has been used for food, specifically as a sweetener, for many centuries. It is a valuable food, prized for its sweetness, aroma and flavor. However, the most recent research demonstrated that honey exhibits several biological functions such as antioxidant, antibacterial and anti-inflammatory properties. The functional properties of honey result from its physico-chemical properties.

## ***1. Physico-chemical properties of honey***

### ***1.1. Sugar content***

In recent years, over 200 compounds have been identified in honey. Sugars are the main constituent, comprising about 95% of honey dry weight. Among the sugars, monosaccharides are the predominant components represented by fructose (38%) and

glucose (31%). The total content of sugars depends on the botanical origin of honey and varies between 60 to 85 %. By comparison, only small amounts of oligosaccharides are present in honey. Disaccharide content (sucrose and maltose) ranges from 7–10%, while trisaccharides (erlose, panose, maltotriose, melezitose) and higher oligosaccharides (isomaltotetraose, isomaltopentaose) account for a total of 3.65% (National Honey Board, 2007; White et al., 1963, Morales et al., 2006 and Astwood et al., 1998).

### ***1.2 Osmotic effect, water activity, and moisture content***

The moisture content of ripened honeys varies from 15 to 21% depending on the honey type. The high concentration of sugars and low moisture content makes honey a supersaturated sugar solution of high osmolarity. Hydrophilic sugars form strong hydrogen bonds with water molecules, leaving only a small amount of unbound, free water to perform other chemical reactions. This free water is called water activity ( $A_w$ ). At low moisture content, the water activity is usually very low in honey and range from 0.5 to 0.6 (Molan, 1996). Honey with an increased water activity above 0.8 is prone to fermentation due to the presence of osmophilic yeast in honey (Snowdon and Cliver, 1996). Therefore, moisture level and water activity are two important quality parameters that contribute to the taste and aroma of honey.

Moreover, the high osmolarity and low water activity are crucial features of honey because they prevent microbial growth (Molan, 1996). These features, together with a production of hydrogen peroxide by honey's glucose oxidase, contribute to its antibacterial effect.

### ***1.3 Color***

Honey color is another quality parameter. Honeys are divided into seven color categories for marketing purposes: water white, extra white, white, extra light amber, light amber, amber and dark amber. In the beekeeping industry, color is measured using

the colorimetric scale of Pfund. Recently, honey color is established using more precise spectrophotometric methods such as described by (Huidobro and Simal., 1984).

Honey color results from its chemical composition. Honey contains carotenoids, terpenoids, polyphenolic acids, flavonoids and amino acids/proteins which are known to possess conjugated double bonds that absorb light in visible range resulting in yellow to red to brown colors.

#### ***1.4 Honey pH***

In general honey is acidic with a pH that ranges from 3.5 to 4.5. According to Bogdanov et al., (2004) and Malika et al., (2005) this low pH is attributed mainly to the presence of gluconic acid which is formed during glucose oxidation. The low pH adds stability against microbial spoilage.

#### ***1.5 Minerals***

Some authors have found that the mineral content influences the color and the taste of honeys: The higher the quantity of metals and the darker color, the stronger is the taste the honey will have (Sancho et al., (1991). Also, Al et al., (2009) found that light coloured honeys usually have low ash content, while dark-coloured honeys generally have a higher ash content. On the other hand, the honey color depends on the geographical region and the mineral availability in the soil. Guizelan et al., (1971) found that the quantity and variety of minerals present in honey depends on the nutrients that have been absorbed by the plants, their availability in the soil or water.

The chemical and physical features of honey are summarized in Table 1.

Table 1. Chemical composition of honey

Component	Average (%)	Range (%)
<b>Monosaccharides:</b>		
Fructose	38.40	30.90-44.30
Glucose	30.30	22.90-40.70
<b>Disaccharides:</b>		
Sucrose	1.30	0.20-7.60
Other disaccharides (maltose, isomaltose, maltulose, turanose and kojibiose)	7.30	2.70-16.0
<b>Oligosaccharides</b>	1.40	0.10-3.80
<b>Water</b>	17.20	12.20-22.90
<b>Gluconic acid</b>	0.57	0.17-1.17
<b>Amino acids:</b> proline, alanine, arginine, serine, glycine, isoleucine, threonine, valine, leucine, glutamic acid, cysteine, phenylalanine, tyrosine, tryptophan, lysine, glutamine, aspartic acid, asparagine, methionine and histidine)	0.43	0.13-0.92
<b>Lactones</b>	0.14	0.0-0.37
<b>Minerals</b> (Potassium, sulfur, chlorine, calcium, phosphorus, magnesium, sodium, iron, copper and manganese)	0.17	0.02-1.03
<b>Nitrogen</b>	0.04	0.0-0.13
<b>Enzymes :</b> Invertase , Amylase, Glucose oxidase, Catalase , and Phosphorylase		0.2-1%
<b>Vitamins:</b> Riboflavin, Pantothenic acid, Niacin, Thiamin, pyridoxin, ascorbic acid		
The pH of honey is ~3.9 and ranges from 3.4-6		

## 1.6 Polyphenols

Polyphenols are the most abundant antioxidants in human diets. They are secondary metabolites of plants. These compounds are designed with an aromatic ring carrying one or more hydroxyl moieties. Several classes can be considered according to the number of phenol rings and to the structural elements that bind these rings (Tsao and McCallum, 2010). In this context, two main groups of polyphenols, termed flavonoids and non-flavonoids, have traditionally adopted.

Flavonoids can be considered as a subgroup of the polyphenols, and they are plant secondary metabolites with a  $C_6-C_3-C_6$  skeletal system. Most encounter flavonoids contain ring structures as shown in Fig 1, although open structures such as the chalcones have traditionally been included in the category because of the similarity in biosynthesis. The chemical structures of the  $C_6-C_3-C_6$  flavonoids are based on a chromane ring (ring C), which most of the time bears a second aromatic ring B in position 2; however, ring B can also be attached to positions 3 (isoflavones) or 4 (neoflavonoids) of ring C (Tsao and McCallum, 2010). Fig 1.

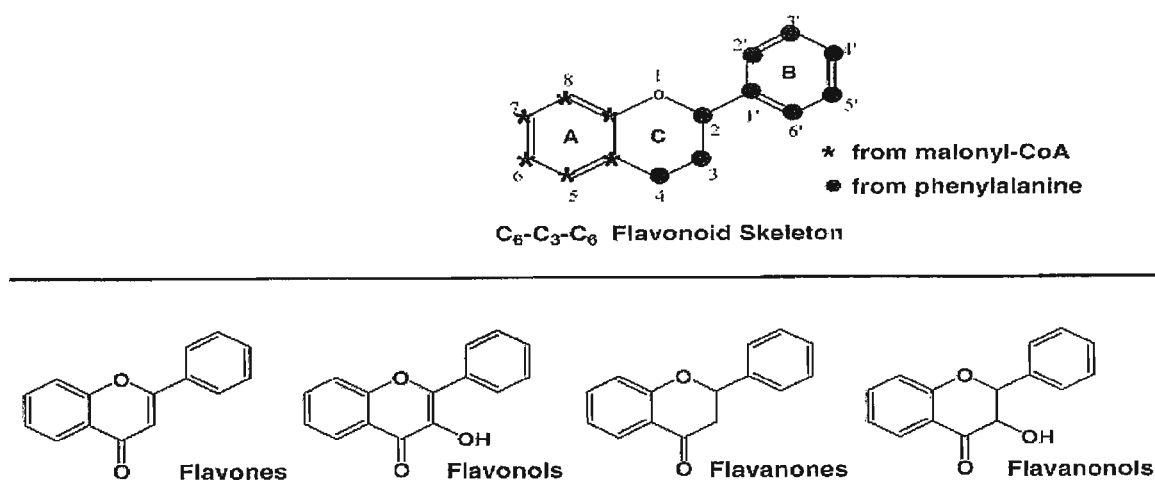


Fig 1. The basic  $C_6-C_3-C_6$  flavonoid skeleton.



Flavonoids are plant pigments chiefly responsible for the red and blue color in fruits, fruit juices, wine and flowers (Havesteen, 2002). Most flavonoids in plant cells are present as glycosides. Sugar substitution on the flavonoid skeleton may occur through hydroxyl groups in the case of O-glycosides. The number of sugar rings substituted on the aglycone varies from one to four (Stobiecki, 2000) Fig 2. In honey, most of the flavonoids are present in glycosidic form as O- glycosides.

Stobiecki, (2000) also stated that flavonoids are structurally a rather diverse group of natural products. The most important variations in their structure arise from the level of oxygenation (hydroxyl or methoxyl groups) and the point of attachment of ring B (flavonoids and isoflavonoids). The sugar component may consist of hexoses, deoxyhexoses or pentoses and in some cases glucuronic acids with the added possibility of O- or C-glycosidation.

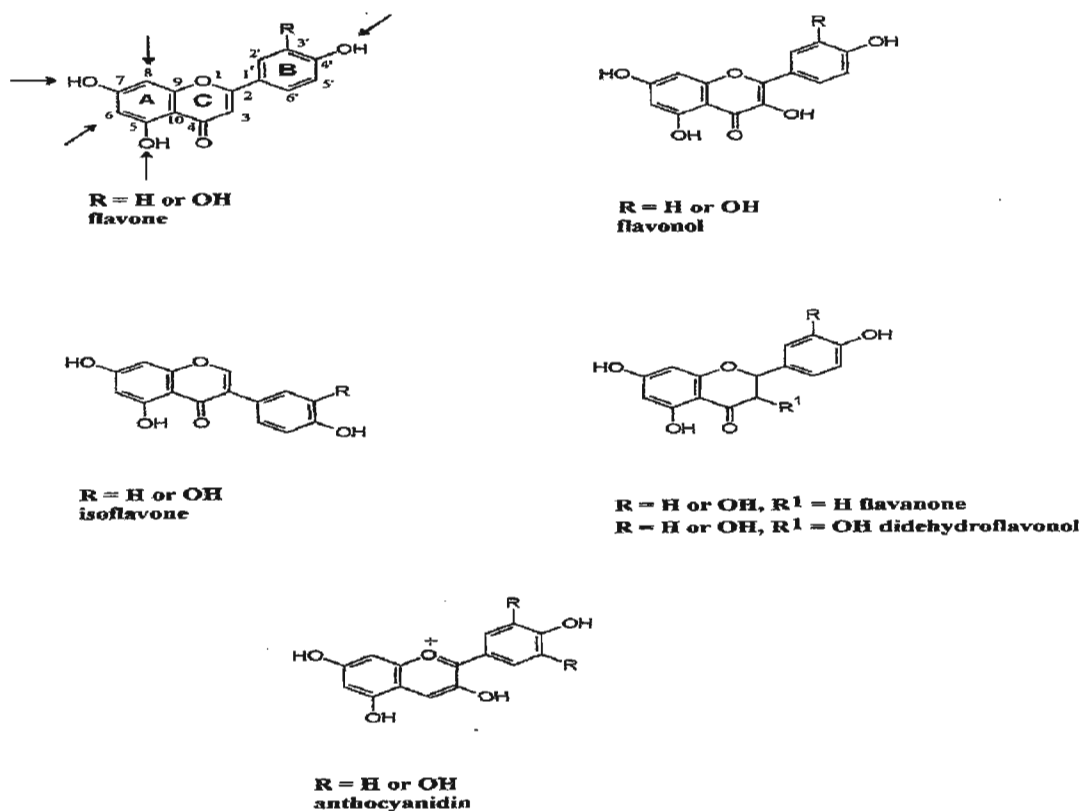


Fig 2. General structure of flavonoids and sites of their glycosylation and methylation, position of possible glycosylation of the flavones aglycone are indicated with arrows.

Nonflavonoids group is classified according to the number of carbons that they have and comprises the following subgroups: simple phenols, benzoic acids, hydrolysable tannins, acetophenones and phenylacetic acids, cinamic acids, coumarin, benzophenones, xanthones, stilbenes, chalcones, lignans and secoiridoids (Tsao and McCallum, 2010).

Phenolic compounds are among the minor constituents in honeys, however, they greatly contribute to honey structure and biological activities. Honey polyphenolic compounds include both phenolic acids and flavonoids (Tomas-Barberan, 2001).

Phenolic acids, of a general structure of  $C_6-C_1$  to  $C_3$ , belong to two groups; hydroxybenzoic acids (benzoic, salicylic, gallic and ellagic acids) and hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric and sinapic acids) (Harborne, 1988). Both of these groups are represented in honey.

The concentration of polyphenols varies in honeys of different botanical origin and ranges from 46.0 to 456 mg/kg (Gheldof et al., 2002). Darker-colored honeys usually contain higher concentrations of polyphenols than lighter-colored honeys, with dark buckwheat honey possessing the highest content. Polyphenols in honey are responsible for its biological functions such as antioxidant activity (Gheldof et al., 2002). The antioxidant activity of polyphenols results from their hydrogen-donating activity, radical scavenging activity as well as their ability to complex divalent transition metal cations. Antioxidant potency depends on the structure of polyphenolic acids and flavonoids such as the presence of catechol structure and the number of hydroxyl groups in the molecules (Rice-Evans et al., 1997). Honey can prevent deteriorative oxidation reactions in foods, such as lipid oxidation in meat and enzymatic browning of fruits and vegetables (Rieck and Dawson, 2000, McKibben and Engeseth, 2002). Honey has, therefore a great potential to serve as a natural food antioxidant.

In addition to antioxidant activity, phenolic acids and flavonoids of honey have been shown to exhibit antibacterial activity (Aljady and Yusoff, 2004, (Bogdanov, 1997, Aljady and Yusoff, 2003, Brudzynski and Miotto, 2010). Gheldof et al., (2002) demonstrated using High Performance Liquid Chromatography (HPLC) and Liquid Chromatography/Mass Spectrometry (LC/MS) that most honeys tested had similar, but quantitatively different, phenolic profiles. However, some phenolic compounds may be uniquely present in honeys of specific botanical origin and serve as a marker.

Table 2 present a list of typical honey flavonoids and polyphenols.

Table 2. Flavonoids and phenolic acids found in honeys

Flavonoids	Phenolic acids
Myricetin	Gallic acid
Rutin	Protocatechuic acid
Kaempferol	p-Hydroxybenzoic acid
Luteolin	Chlorogenic acid
Apigenin	Vanillic acid
Tricetin	Caffeic acid
Quercetin	Syringic acid
Pinobanksin	p-Coumaric acid
Flavone	Ferrulic acid
Chrysin	Benzoic acid
Pinoncembrin	Cinnamic acid
Galangin	Hydroxycinnamic acid
Pinostrobin	

Polyphenols contribute to honey color. Literature data indicate that there is a strong positive correlation between honey color and total phenolic content (Beretta et al., 2005; Frankel et al., 1998, Gheldof et al, 2003, Brudzynski and Miotto, 2010). Dark color honeys possess a higher total phenolic content than lighter honeys.

In the past several years, there has been increasing evidence that flavonoids of fruits and vegetables are propitious to human health (Merken and Beecher, 2000) as a result of their anti-allergic, anti-inflammatory, anti-viral, anti-cancer and anti-AIDS, anti-mutagenic and antihypertensive properties (Hertog et al., 1992; Middleton, 1996; Plessi et al., 1998; Robards and Antolovich, 1997). More studies on honey polyphenols are needed to fully understand their biological activity and their effect on human health.

### ***1.7 Honey proteins***

The concentration of proteins and amino acids in honeys varies, depending on the botanical, geographical origin and storage time. Protein content ranges from 0.2 to 0.4 mg/100g (Bogdanov, 2010). Using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) it has been found that part of the protein pattern, a number of proteins and polypeptides, is similar for a large number of honeys. Some authors have stated that a similarity of protein profiles in honeys result from the fact that the majority of proteins consist of bee-originated enzymes introduced to honey by bees from their hypopharyngeal glands during honey harvesting (White and Kushnir, 1966). In contrast, recent data indicate that honey also possesses other enzymes and proteins that are of pollen and nectar origin. In addition, one of the major proteins in honey seemed to be identical to royal jelly albumin, Major Royal Jelly Proteins 1 (Simuth et al., 2009). Apalbumin, Apal is a dominant glycoprotein of RJ with a mass of 55kDa (Schmitzova et al., 1998, Hanes and Simuth, 1992) that has been found to be a regular component of honey Simuth et al., (2004).

Apal occupies an exclusive position because it is simultaneously synthesized in honey brain Kucharski et al., (1998) as well as in hypopharyngeal glands of adult honey bee (Hanes and Simuth, 1992, Kubo et al., 1996). It was also demonstrated that the function of Apal in honeys was not limited to nutritional value. Majtan et al., (2005) confirmed that Apal, present in honey, is responsible for activation of murine macrophages and production of  $\text{TNF}\alpha$  (Tumor Necrosis Factor).

Altogether, 9 to 19 different protein bands have been observed using the SDS-PAGE method in unconcentrated native honeys (White and Kushnir, 1966, Marshall and Williams, 1987).

In honey, the enzymes and proteins of bee-origin exceed the amount of proteins coming from pollen (Baroni et al., 2002). The majority of these enzymes are involved in sugar metabolism. The list include; diastase, invertase,  $\alpha$ - and  $\beta$ -glucosidases, glucose oxidase, apalbumin and glucose transferase (White, 1975). Simuth et al., 2009) stated that proteins such as glucose oxidase,  $\alpha$  and  $\beta$  glucosidase and amylase are regular components in honeys. Even if enzymes are added to honeys mostly by bees, the various honey types show considerable differences in enzyme activities (Persano Oddo et al., 1990; Persano Oddo et al., 1999; Persano Oddo and Piro, 2004), most likely due to the rate of the nectar flow and to the physiological stage of the bees' glands during the productive season (Bogdanov et al., 2004).

Diastase is a traditional name of amylase- a plant enzyme involved in hydrolysis of starch to dextrin and to maltose. Diastase levels in honey are parameters of honey quality; its freshness (Gomes et al., 2009). The diastase content varies according to the floral source, long periods of storage and exposure to high temperatures (Boukraa et al., 2008).

Three kind of Invertase ( $\alpha$ -glucosidase, saccharase) have been found in honeybees but only one is secreted into the honey by the bees. Kubota et al., (2004) showed that honey  $\alpha$ -glucosidase was immunologically confirmed to be  $\alpha$ -glucosidase III secreted into the honey from the hypopharyngeal gland.

Invertase catalyses the conversion of sucrose to glucose and fructose. It catalyses also many other sugar conversions and is mainly responsible for honey's sugar pattern (Raude-Roberg, 1994).

A hydrolytic action of amylases and glucosidases leads to the degradation of oligosaccharides to monosacchrides. This sugar degradation is a main reaction during honey ripening and results in high amounts of free glucose and fructose. At the same time, honey ripening is associated with a gradual decrease in water content.

Bees accelerate water evaporation by flapping their wings over the honey cell. In the end of the process of converting nectar to honey, the concentrated sugar solution is created which helps preserve honey against bacterial and fungal spoilage and yeast fermentation.

### ***1.8 Hydrogen peroxide production by glucose oxidase***

Two other main enzymes, glucose oxidase and catalase regulate the production of  $H_2O_2$ , one of the honey antibacterial factors (White et al., 1975, Weston et al., 1999).

Glucose-1-oxidase GOX is a well characterized glycoprotein consisting of two identical 80-kDa subunits with the two FAD co-enzymes bound (Wong et al., 2008). This enzyme catalyses the oxidation of  $\beta$ -d-glucose to D-gluconolactone and hydrogen peroxide. The function of glucose oxidase in honey is not only to metabolize carbohydrates, which helps to maintain the nest homeostasis, but also acts as a defense against plant alkaloids present in the nectars.

Liu et al., (2005) reported that (GOX) helps arthropods deactivate alkaloids (Musser et al., 2002). Musser et al., (2002) demonstrated that the by-products of GOX, hydrogen peroxide ( $H_2O_2$ ), and gluconic acid appeared to be responsible for the suppression of the inducible nicotine effect in wounded tobacco.

According to Shi and Di (2000) substantial amount of  $H_2O_2$ , produced as a by-product of glucose oxidation can deactivate phenolics by forming irreversible complexes with herbivores' enzymes. This raises the possibility that GOX may be inhibited by phenolics in nectar outside the hive. The enzyme catalase, on the other hand breaks down the  $H_2O_2$ . The ratio of glucose oxidase to catalase, regulates the levels of hydrogen peroxide in honey (Weston, 1999).

### ***1.9 Amino acids***

The amino acid content in honey is very low and ranges from 34.19 to 183.16mg/100g, (Cotte et al., 2004; Iglesias et al., 2004). Proline is the most abundant amino acid in honey (Iglesias et al., 2006). Its levels correspond to approximately 33% of the total free amino acid content. Proline is mainly derived from bees' secretions (Von der Ohe et al., 1991). In addition other amino acids present in honey include those presented in Table 3 (Iglesias et al., 2006).

Table 3. Mean values of concentration of Amino Acids found in blend honeys

Amino acids	Conc. (mg/100g of dry matter).
Asp	9.13
Glu	12.89
Asn	11.65
Ser	3.67
Gln	5.96
His	1.31
Gly	1.72
Thr	2.31
Arg	3.49
$\alpha$ -Ala	2.64
$\beta$ -Ala	4.65
Gaba <sup>b</sup>	3.10
Tyr	7.46
Met	0.33
Val	2.34
Trp	3.06
Phe	13.68
Ile	2.11
Leu	2.13
Orn	2.32
Lys	2.75
Pro	89.32

During the period of time in which the bees are working on the gathered nectar the enzymatic activity and the amount of proline is increasing (Von der Ohe, 1994). Proline is an especially important amino acid for insects. It is the most abundant amino acid in the bee hemolymph. In studies, using tethered honeybees, proline is selectively degraded in the hemolymph, and it has been proposed that proline is used by foragers in the initial stages or lift phase of flight (Auerswald et al., 1998; Micheu et al., 2000). Proline is also required for egg-laying by the queen (Hrassing et al., 2003). Thus proline is specifically required for insect development and flight. This explains why the amino acid, proline, is the most abundant in honeys (Carter et al., 2006).



The oxidative proline degradation pathway utilizes proline as a source of energy, specifically during the initial "lift" phase of insect flight. Proline is rapidly metabolized and results in the production of multiple nicotinamide adenine dinucleotide phosphate (reduced form) equivalents and high levels of adenosine triphosphate (ATP). No other amino acid can be metabolized as rapidly as proline and release as much ATP without complete metabolism. While glucose ultimately yields more ATP on a molar basis the initial steps of glucose metabolism requires the consumption of ATP. Thus proline is a more efficient fuel in the short run, while glucose is a far superior fuel in the long run. However, proline is metabolically more expensive than glucose for the plant the plant to produce. Glucose can be produced from atmospheric gases by photosynthesis whereas proline cannot (Carter et al., 2006).

#### ***1.10 Protein– polyphenol complexes***

Protein–polyphenol complexes are believed to play a role in plant defense mechanisms against herbivorous ingestion due to their unpalatable and antinutritional properties. Underlying this phenomenon is the polyphenols distinctive ability to form intermolecular complexes with each other and with other molecules. It has been stated that most predominant characteristic of polyphenols is their affinity for proteins. Thus, many of their actions appear to depend, either directly or indirectly, on this ability, which leads to the formation of soluble or insoluble complexes. (Manchado–Sarni et al., 2008).

The formation of protein–polyphenols complexes has been largely studied in saliva, specifically between the proline– rich proteins of saliva and polyphenols of beverages such as beers and wine. Several models have been reported for the possible protein–polyphenol complex formation. Rawel et al., (2005) stated that, because the polyphenols are small molecules (180–700kDa) and proteins are comparatively very

large (14000–350000kDa) it is possible that more than one polyphenol molecule bind to one protein molecule. The average ratio of proteins to polyphenols is 1:60 to 1:100.

The evidence further suggests that there is a critical level of coating of the peptide by polyphenol and that when this level is exceeded, the complex precipitates (Charlton et al., 2002).

Bennick, (2002) states that the nature of tannin–protein interaction has been the subject of many studies. Potentially, such interactions could occur via covalent or ionic binds, hydrophobic interaction, or hydrogen bonding. While polyphenols are prone to oxidation and give rise to ortho–quinones which are highly reactive intermediates that potentially could result in tannin–protein crosslinks (Haslam et al., 1991), there is at present little evidence for covalent binding of tannin to protein. No interaction has been observed of tannin and protein at pH values where the phenolic hydroxyl groups in tannin would be ionized, indicating that ionic interaction with protein is of little or no importance (Hagerman and Butler, 1978).

Early studies of polyphenol/protein binding suggested that polyphenols bound preferentially to proline residues (Hagerman and Butler, 1981). However, proline is certainly not the only possible binding site. For example it has been suggested that polyphenols bind tightly to histatin, which are salivary proteins containing a high proportion of histidine residues (Yan and Bennick, 1995; Naurato et al., 1999).

### ***1.11 Honey as food for larvae***

Honey is a high–energy food for bee larvae that takes up a minimum area in the honey comb (Crane, 1975 and Crane, 1990). Together with honey proteins, as a main source of nitrogen, vitamins and minerals honey seems to be a fully nutritive food for bee progeny.

In hives, honey is kept in air-tight wax compartments and sealed with a propolis cap. As a supersaturated sugar solution with low water activity, honey is preserved to withstand long storage without diminishing its nutritional value.

### ***1.12 Honey elaboration (maturation/ripening)***

The chemical composition of nectar is similar to that of honey and includes sucrose, glucose and fructose, organic acids (Baker and Baker, 1975), terpenes (Ecroyd et al., 1995), alkaloids (Deizer et al., 1977), flavonoids (Rodriguez-Arce and Diaz, 1992), glycosides (Roshchina and Roshchina, 1993), vitamins (Carter and Thornburg 2004a; Grielbel and Hess, 1940), phenolics (Ferrerres et al., 1996), and oils (Vogel, 1969). Nectars also contain specific plant-defense proteins that appear to function in the protection of the gynoecium from microbial invasion (Carter and Thornburg, 2000, 2004a, b, c; Carter et al., 1999, Naqvi et al., 2005, Peumas et al., 1997, Thornburg et al., 2003). Honey elaboration, that is, transformation from nectar to the final honey product, occurs in several steps. During this conversion, several changes take place.

Firstly, after the nectar is collected by the bees, the most prominent step in the maturation process is the considerable water loss (40 to 70 % of nectar initial weight). Water loss takes place in two stages: an initial evaporation carried out by the bee, which brings water content down to 40 to 50%, and the final evaporation that takes place in the honeycomb, which yields a product with 15 to 18% water. (Arguezo-Ruiz and Rodriguez-Navarro, 1975).

Secondly, nectar oligomeric sugars are externally inverted as a result of carbohydrate-metabolizing enzymes secreted by the worker bees' hypopharyngeal glands (Ohasi et al., 1999).

Thirdly, there is a marked decrease in the pollen grains during honey elaboration (Von der Ohe, (1994). As the honeybee visits the flower in hunt of nectar, some of the

flower's pollen falls into the nectar collected by the bee and stored in the stomach which will be regurgitated along with the nectar. Moreover, some pollen grains often attach themselves to various parts of the honeybee body like legs, antenna, hairs, and also in the eyes of visiting bees. This pollen will then get entangled in the hive and thereby gain entry into the honey. It has been reported that ten grams of honey contains 20 to 100 000 grains of pollen (Jaganathan and Mandal, 2009; Bauer et al., 1996). An enormous reduction in pollen takes place during honey ripening (Von der Ohe, 1994). Von der Ohe (1994) found that there is a correlation between the reduction in the amount of pollen and the increase in proteolytic enzyme activity or between the reduction of pollen and the increase in amount of proline.

Furthermore, the homeostatic conditions in bee colonies, in which the levels of CO<sub>2</sub> and the temperature of 35 °C remain stable, seemed to accelerate phenolic compounds degradation. Liu et al., (2005) showed that the highly controlled nest homeostasis facilitates direct deactivation of phenolics in nectar, and plays a role in the action of hypopharyngeal glands proteins (HGP) as well. These conditions seemed to prevent the formation of irreversible complexes of phenolics with proteins (Shi and Di 2000 ; Liu et al., 2005).

Liu et al., (2005) demonstrated that Glucose oxidase from HPG is inhibited outside the hive and the phenolic content was significantly reduced inside of the hive. It was found that the large reduction of phenolics in nectar with HGP (hypopharyngeal gland proteins) in the hive ( $0.6490 - 0.3667 = 0.2823$ mg/ml) reflects the contribution of several factors, including the breakdown in the nest environment (0.1623mg/ml), the precipitation of phenolics by HGP and a the deactivation of phenolics by GOX. Liu et al., (2005). According to the results obtained by (Musser et al., 2005) in their research about the caterpillar salivary enzyme, glucose oxidase, and its role in the defense against plants alkaloids, they found hydrogen peroxide to activate the WIPK, a Mitogen-

activated protein (MAP-kinase) involved in the regulation of systemic acquired resistance (SAR) to pathogens in tobacco (Romeis et al., 1999 ; Seo et al., 1995). A potential mechanism for the suppression of nicotine by GOX is through the induction of Salicylic acid (SA) by  $H_2O_2$ , a by product of GOX activity (Bi et al., 1995 ; Wu et al., 1995 ; Alvarez et al., 1998 ; Chamnongpol et al., 1998).

Considerable microbial populations exist in the initial stages of honey maturation and may be involved in some of these transformations (Argueso-Ruiz and Rodriguez-Navarro, 1975; Argueso-Ruiz and Rodriguez-Navarro, 1973).

All microorganisms associated with ripening honey are also found in the bee. Gluconobacter and lactobacillus populations decrease as ripening proceeds; the number of bacteria gradually decreases from nectar to higher-moisture honey to low-moisture honey (Argueso-Ruiz and Rodriguez-Navarro, 1975).

### ***1.13 Effect of post-harvest processing and environmental factors on the honey composition and quality***

It is in generally understood that the quality of the honeys decreases with the time of storage. Temperature, light and humidity all contribute to changes in the chemical composition and organoleptic characteristics of honeys.

The most prominent change includes the acceleration of the Maillard reaction which causes browning of honey and an increase in the levels of hydroxymethylfurfural, a product of fructose dehydration. Due to the toxicity of hydroxymethylfurfural, its levels are strictly limited in honey (Codex Alimentarius, 2001).

As the protein and enzyme concentration in honey decreases with storage, enzyme activity is often used as an indication of freshness in honeys. The freshness of honey is described as "diastase number" and along with Invertase activity is used to

measure quality of honey. Invertase activity together with diastase activity and hydromethylfurfural content, is a honey quality control parameter (Sanchez et al., 2001).

**Objectives:**

Proteins are minor components of honey and as such have not been given sufficient consideration in previous research. To assess their role in biological activities of honey, it is important to investigate their profiles in different honeys, their concentration, interaction with other honey components, specifically with polyphenols, and changes in their levels during honey storage.

Therefore, the objectives of this study are as follow:

1. To determine the protein profiles and their concentrations in honeys from different botanical origins.
2. To determine the changes in protein profile and protein concentration with storage time.
3. To investigate potential interactions between proteins and polyphenols.

## 2. Materials and Methods

### 2.1 Honeys

Honeys were obtained directly from beekeepers and included both commercial (pasteurized) and apiary samples (raw honeys). Honeys originated from Ontario, Alberta, and British Columbia, Canada. These honeys were kept at room temperature and in the dark, Table 4.

Table 4. Provides a list of honeys, their botanical origin, province, and year of harvest

Honey	Botanical origin	Province	Year of harvest
76	buckwheat	BC	2008
77	buckwheat	Alberta	2008
99	sunflower	BC	2008
109	cranberry	BC	2008
114	sunflower	BC	2008
65	pumpkin	BC	2008
66	blackberry	BC	2008
67	blueberry	BC	2008
147 (M)	manuka	New Zealand	2008
125	buckwheat	BC	2008
177	buckwheat	ON	2009
178	blue berry	ON	2009
179	Wild flower	ON	2009

## ***2.2. Honey color determination***

The intensity of color of honey was measured spectrophotometrically and presented as the net absorbance at  $A_{(560-720\text{ nm})}$ , according to the method of Huidobro and Simal, (1984).

## ***2.3. pH***

A solution of 50% (v/v) honey was prepared to measure the pH. The solution was made by mixing 1 ml of liquid honey with 1 ml of distilled water or by weighing 1.75 g of crystallized honey and dissolving it in warmed, distilled water (1 ml of liquid honey weight approximately 1.75g). The pH was measured using a Symphony SB80PC pH meter.

## ***2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-***

### ***PAGE)***

SDS-PAGE was performed according to the method described by Laemmli (1970). Honey proteins were analyzed on 7.5% gel separation gel with attached 5% stacking gel. 30  $\mu$ l of a honey solution (50% v/v) was mixed with 10  $\mu$ l of sample buffer and loaded on a gel. Sample gel contained: 3M tris(hydroxymethyl) aminomethane (Tris) pH 8.8, 10% SDS, 30%:1% Acrylamide:bis-acrylamide, 0.5M Ethylenediaminetetraacetic acid (EDTA), H<sub>2</sub>O, 10% Ammonium persulfate (APS) and 10% Tetramethylethylenediamine (TEMED). The electrophoresis was carried out at a constant current of 140 mA through the stacking gel, and at 140 mA through the separation gel. SDS-PAGE was conducted at room temperature using a Mini Protean III electrophoresis cell (Bio-Rad Laboratories, Hercules CA). After electrophoresis, gels were fixed in a solution of acetic acid and methanol (1:1), washed in distilled water and stained either with Coomassie Brilliant Blue R-250 (Bio-Rad) or Silver Stain (Bio-Rad Silver Stain Kit).



The molecular weight of the proteins were determined from the plot of the log MW versus relative mobility of the bands, using molecular weight standards (Fermentas Life Sciences, PageRuler Prestained Protein Ladder #SM0671).

### ***2.5. Determination of Protein Concentration***

The protein concentration in honeys was determined by Pierce BCA Protein Assay Kit. The Pierce BCA protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well known reduction of  $\text{Cu}^{+2}$  to  $\text{Cu}^{+1}$  by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of cuprous cation ( $\text{Cu}^{+2}$ ) using the unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentration over a broad working range (20–2,000 $\mu\text{g}/\text{ml}$ ). The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids, (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for the color formation with BCA. The protein concentration generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentrations were used to produce the standard curve.

### ***2.6. Proteolytic degradation***

To test whether the reduction of protein concentration was due to proteolytic degradation in our honeys, honeys H178 and H147 were incubated with bovine serum albumin (BSA), of known concentration (2mg/ml). The mixtures were incubated for 15, 30 minutes and 1 hour, then they were analyzed for the presence of BSA degradation products using SDS-PAGE as described previously.

## 2.7. Scion Imaging System

Scion imaging software (Scion Corporation Release 4.0.3.2) was applied to determine protein concentration in gels. The concentration of proteins was quantified by comparing the number of pixels appearing in the protein band area to the number of pixels obtain from BSA band of known concentration.

## 2.8 Protein and phenolic extraction

Two protocols were designed to separate protein from polyphenols. Protocol 1 (P1) and Protocol 2 (P2), Fig 3.

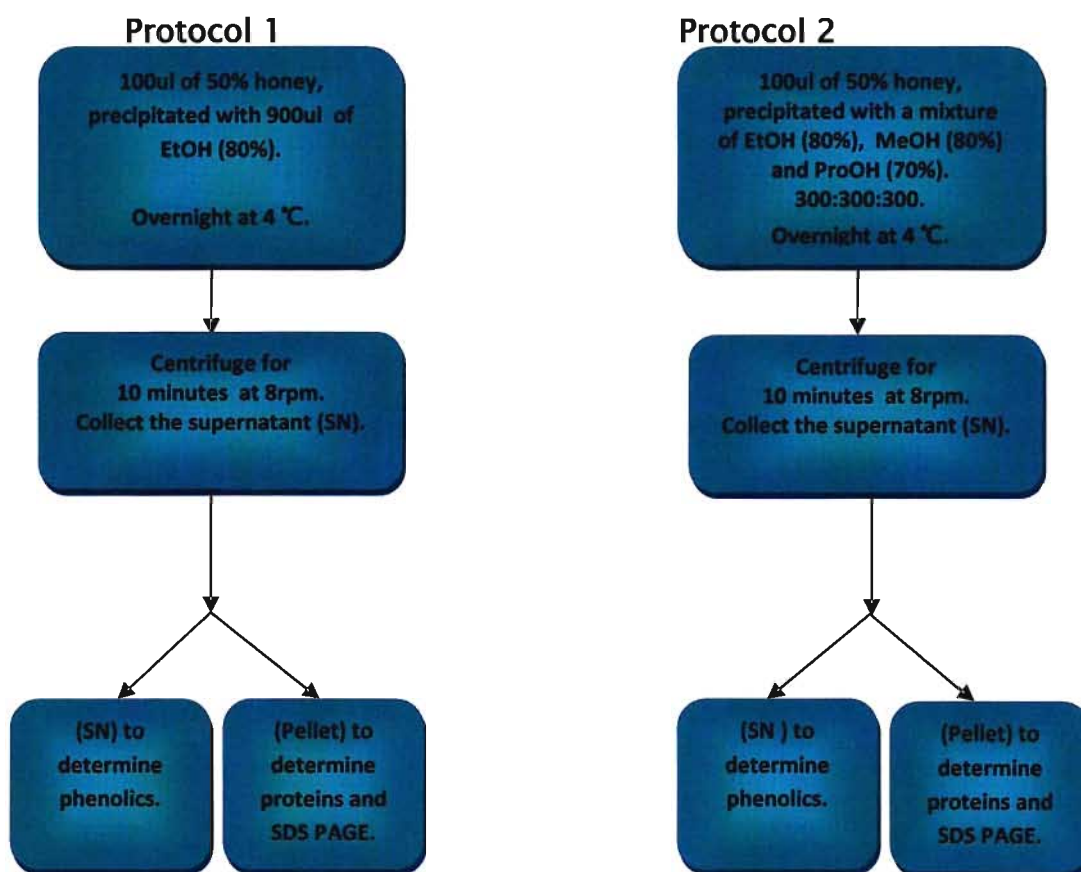


Fig 3. Protocols 1 and 2, designed to separate proteins from polyphenols.

### ***2.9 Determination of Total Phenolic Content***

The total phenolic content was determined using the Folin–Ciocalteu method modified by Singleton and Rossi, (1965). 200µl of the sample (50% honey solution or a supernatant obtained after protein precipitation) was mixed with 2.4ml of the freshly prepared working solution of Folin–Ciocalteu reagent. Working solution was prepared by diluting the concentrated Folin–Ciocalteu reagent 1:17 with distilled water. After 1 minute, 420µL of sodium bicarbonate (20 % (w/v) was added and the mixture was allowed to stand for 1 h at room temperature, in the dark. After incubation, 910 µL of distilled water was added to each tube. Absorbance was measured at 765 nm.

The same procedure was employed to establish a standard curved using gallic acid solutions (31.2, 62.5, 125, 250, 500µg/ml). The total phenolic content was expressed µg gallic acid equivalents (GAE) per g of honey.

### ***2.10. Solid Phase Extraction (SPE)***

Solid Phase Extraction was used to remove interfering substances and impurities from samples before the determination of the total phenolic content by the Folin–Ciocalteu method and before LC/MS analysis. Waters Oasis HLB 3cc Extraction Cartridges (Oasis HLB from Waters, Milford, MA) were mounted on a manifold attached to the vacuum pump and conditioned and equilibrated by passing 1 ml of 100% methanol, followed by 1 ml of distilled water. 1 ml of 50% diluted honey sample was loaded on the cartridge, washed with 1 ml of 5% methanol. The phenolic compounds remained in the column while sugars and other polar compounds eluted with the aqueous solvent. 1ml of methanol 100% was added to elute the phenolic compounds. The whole phenolic fractions were evaporated in a dry bath and reconstituted in 1 ml of distilled water to be analyzed.

### ***2.11. Lyophilization***

The methanol eluates from SPE were lyophilized using a speedvac (Savant, Thermo Scientific, USA), reconstituted in 50 $\mu$ L of methanol and subjected to LC/MS analysis.

### ***2.12. Polyphenol extraction and determination using Liquid Chromatography***

#### ***Mass Spectrometry (LC/MS)***

Honey (50%) solutions were purified using solid phase extraction and subjected to liquid-liquid extraction in the following order: (a) Protocol 2, mixture of 300ml of ethanol (80%), 300ml of methanol (80%) and 300ml of isopropanol (70%) and (b) acetone. Ultimately, the procedure provided three polyphenol fractions: total phenolic fraction, free phenolic fraction after extraction with mixture of three alcohols (Protocol 2), and bound phenolic fractions after extraction with acetone. At each extraction step, removed polyphenols (supernatant fraction) were collected, lyophilized and reconstituted in methanol for LC/MS analysis, Fig 4.

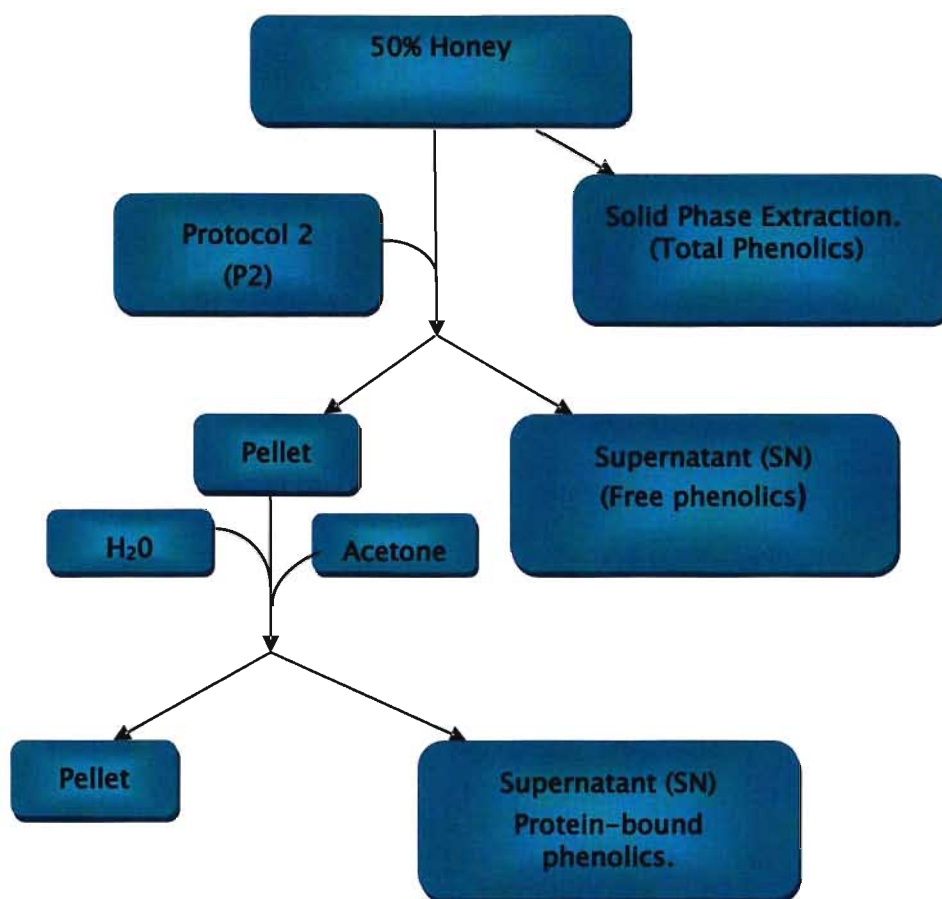


Fig 4. Steps of polyphenol extraction by different methods, Protocol 2, Solid Phase Extraction and treatment with acetone.

LC/MS was performed at the Brock University MS facility with a Bruker HCT Ultra LC/MS instrument. The equipment was conformed first using the UV/MS detection serial on-line detection first by UV at 254nm followed by MS. Polyphenols were separated using a ZORBAX Eclipse XDB- C18, 4.6 x 50 mm column (Agilent) using a isocratic gradient developed from A: 2mmol/L formic acid (pH 2.7) and B: methanol.

Elution gradient time: 0–3min	22%B
10min	100%B
12min	100%B
13min	22%B
16min	22%B

ESI was run with negative ion polarity with a capillarity exit voltage of –128.5 Volts. The elutions were monitored at 254 nm for a total run time (RT) of 29 minutes.

The several flavonoids and polyphenolic acid standards, typically present in honey, were resolved under the above LC-ESI/MS conditions to obtain their retention times (RT) and mass ions (Table 5 and 6). Identification of phenolics was carried out by comparing retention time and spectral characteristic of unknown analytes and standards.

Table 5. Retention times and mass ion of polyphenolic acids standards

Standard	Retention time (min)	(M-H) (m/z)
Gallic acid	1.13	169
Protocatechuic acid	1.9	153
Chlorogenic acid	3.1-3.3	353
Caffeic acid	3.6	179
p-coumaric acid	5.3	163
Ferulic acid	5.9	193
Cinnamic acid	11.1	147

Table 6. Retention times and mass ion of flavonoid standards

Name	Retention Time (min)	Mass ion (m/z)
Rutin	8.0	609
Myricetin	11.0	317
Quercetin	11.8	301
Luteolin	12.5	285
Hesperitin	13	301
Pinobanksin	13.15	271
Apigenin	13.2	269
Kaempferol	14.19	299
Pinocembrin	17.4	255
Pinostrobin	18.2	269

### *2.13. Statistical analysis*

The statistical program GraphPad InStat version 3.05 (GraphPad Software Inc.) was used to perform statistical analyses. Data were analyzed using a one-way ANOVA and Tukey–Kramer Multiple Comparison test or an unpaired t-test. Differences between means were considered to be significant at  $p < 0.05$ .

## **3. Results**

### **PART I**

The specific aim of Part I of the thesis was to investigate protein profiles in honeys of different botanical origins and to assess stability of the protein pattern during honey storage.

#### ***3.1 General characterization of honeys***

A set of 13 freshly received, raw honeys was used in the study. These honeys originated from different botanical sources and were visibly different in their color (Table 7). Honeys were characterized based on their color, pH and protein profile.

##### ***3.1.1. pH***

The pH is a very important parameter which influences honey color, texture, the stability of constituents and the shelf life of honeys. The pH measurements were conducted on undiluted, freshly obtained honeys. As shown in Table 7, pH ranged between 3.59 to 4.83. These results are in good agreement with that of literature data (Malika, Mohamed and Chakib, (2005). Light color honeys showed more alkaline pH values and the darker ones more acidic. The acidic pH in honeys depends on the content of gluconic acid produced mainly by the enzyme glucose oxidase during glucose oxidation. In addition, other non-aromatic- (formic, acetic acids) and aromatic acids (hydroxybenzoic and hydroxycinnamic group of acids) influence the final pH of honeys.

##### ***3.1.2 Color determination***

Honeys from different botanical origin possess different compositions and concentrations of pigments (mostly polyphenols and carotenoids). Due to these differences, honeys differ in colors, varying from dark brown to almost white.



Color is an important quality parameter and is assessed by beekeepers using the Pfund scale. However, this method is imprecise. In our laboratory, honey color has been routinely determined using the spectrophotometric method of Huidobro and Simal, (1984). The color is expressed as the net absorbance obtained at two wavelengths ( $A_{560}-A_{720}$  nm). The color of honeys measured by this method ranged from 1.88 to 0.085 absorbance units (au), with buckwheat honey being the darkest and pumpkin-the lightest.

Table 7. Physical characteristics of the honeys

Honeys	Intensity of Color * (Net Absorbance) ( $A_{560}-A_{720nm}$ )	pH	Botanical Origin	Year
76	0.965	3.59	buckwheat	2008
77	1.886	3.81	buckwheat	2008
99	0.298	3.76	sunflower	2008
109	0.151	3.77	cranberry	2008
114	0.172	3.44	sunflower	2008
65	0.085	3.45	pumpkin	2008
66	0.096	3.40	blackberry	2008
67	0.101	3.50	blueberry	2008
147 (M)	0.539	3.86	manuka	2008
125	1.880	3.76	buckwheat	2008
177	0.66	3.77	buckwheat	2009
178	0.165	4.83	blueberry	2009
179	0.131	4.07	wild flower	2009

\* Intensity of color (net absorbance) is expressed in au

### ***3.1.3. Protein profiles and content in different honeys***

Proteins comprise only a small portion of total constituents in honeys (0.5 to 1%) Saravana and Mandal, (2009). Proteins in honey derive from different natural sources such as bee pollen, nectars and from honey bees themselves. Protein profiles in honeys seem to be similar due to predominant amount of enzymes secreted to honey from bee hypopharyngeal glands such as amylase, glucose oxidase, glucosidases, and invertases.

Protein profiles of Canadian honeys have not been analyzed before. It was expected that the protein profile would vary with botanical origin of pollen and nectar.

To determine protein patterns in honeys, 1D- SDS-PAGE was employed. The proteins were separated on a 7.5% gel, stained with Coomassie Blue and the obtained protein profiles were compared between honeys. As shown in Fig. 5, the protein profiles differed among the tested honeys in the number and intensity of bands. One to 8 bands were observed in honeys tested. The most consistent and abundant protein bands were observed in the range of 95 to 55 kDa (Fig. 5). In addition, the polypeptides bands differed in their intensity of staining, indicating differences in polypeptide concentrations.

Three different protein patterns were observed; a long protein profile, consisting of 7 to 8 protein bands (Honeys 76, 77 and 99), a medium- length profile for honeys (67, 109 and 114) and a short profile for honeys (65 and 66). It has been observed that honeys from the same botanical source, seemed to have similar protein profiles, such as in the case of honeys originated from buckwheat H76, H77 and sunflower H99 and H114 (Fig.5).

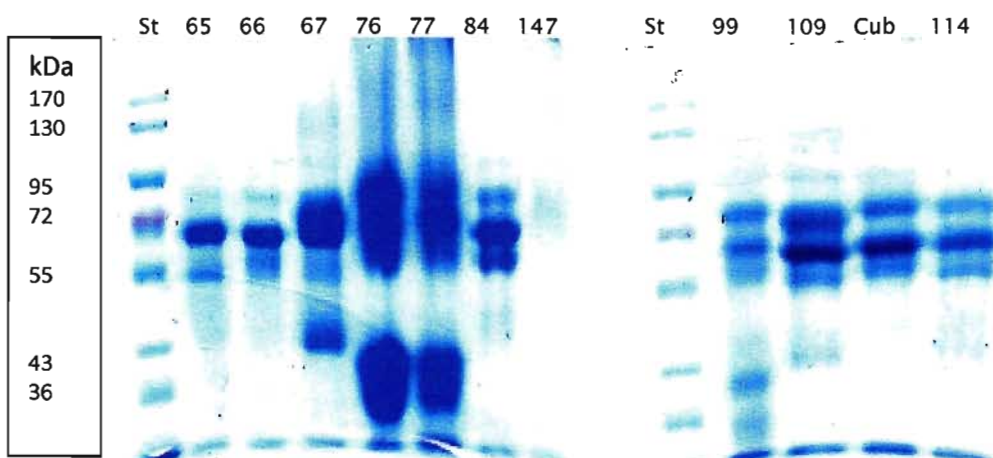


Fig 5. Protein pattern in honeys from different botanical origin

#### 3.1.4. *Molecular size of protein present in different honeys*

The estimated molecular size of proteins was established using the Molecular Size Ladder (Fermentas Life Sciences), containing pre-stained protein standards. The standard curved was constructed by plotting the relative mobility of the protein in the gel versus the log of its molecular weight (MW). Fig. 6 represents the standard curve constructed on data from ten independent experiments.

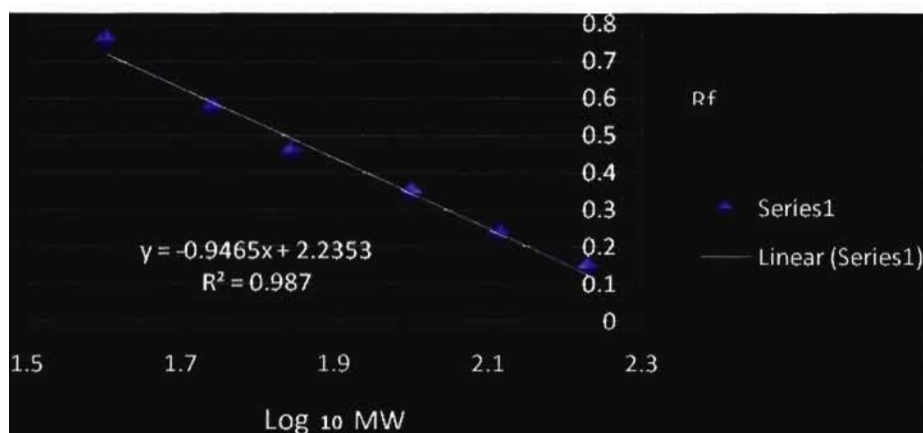


Fig 6. Standard curve. Protein Ladder (Fermentas)

The estimated molecular size of proteins in honeys ranged from 130 to 25 kDa (Table 8). The bands observed were of 97, 84, 70, 60, 52, 39, 32 and 25kDa (Table 8 and 9). All honeys shared three to four common bands: 84, 70, 60 and 52kDa (Table 8).

**Table 8. Common, shared protein bands in different honeys**

H76 buckwheat	H77 buckwheat	H99 sunflower	H109 cranberry	H114 sunflower	H125 buckwheat	H147 manuka
97	97	97	97	97	97	
<b>84</b>	<b>84</b>	<b>84</b>	<b>84</b>	<b>84</b>	<b>84</b>	
<b>70</b>	<b>70</b>	<b>70</b>	<b>70</b>	<b>78</b>	<b>70</b>	72
<b>60</b>	<b>60</b>	<b>60</b>	<b>60</b>	<b>60</b>	<b>60</b>	—
<b>52</b>	<b>52</b>	<b>52</b>	<b>52</b>	<b>52</b>	<b>52</b>	55
39	39	39	—	39	39	43
32	32	32	—	32	32	
25	25	25	25	25	25	25

**Table 9. Molecular sizes in protein bands present in honeys**

H65 pumpkin	H66 blackberry	H67 blueberry
—	—	—
—	—	130
—	—	—
<b>82</b>	<b>82</b>	<b>82</b>
<b>70</b>	<b>70</b>	<b>70</b>
—	—	—
<b>60</b>	<b>60</b>	<b>60</b>
<b>52</b>	<b>52</b>	<b>46</b>
25	25	25

### *3.2 Changes in protein profile with the honey storage*

As we continued monitoring the protein pattern in different honeys, it was noticed that there were changes in the intensity of staining of some bands during honey storage. The protein bands on SDS gels became visibly diffused, some bands disappeared and the background staining increased between the bands. Fig. 7A and B compares the protein profiles of the same honeys obtained before and after two months in storage. The fuzzy bands and sharp decrease in the intensity of band staining were specifically visible in dark buckwheat honeys H76, H77, and sunflower honeys H99 and H114. Fig 7C and D. In the case of honey H114 the almost complete disappearance of

bands was observed. The changes in the protein profiles were not due to technical problems with the running of SDS-PAGE. They appeared repeatedly in stored honeys, and became a point of interest in this study.

These results suggested that proteins in honey were very sensitive to storage.

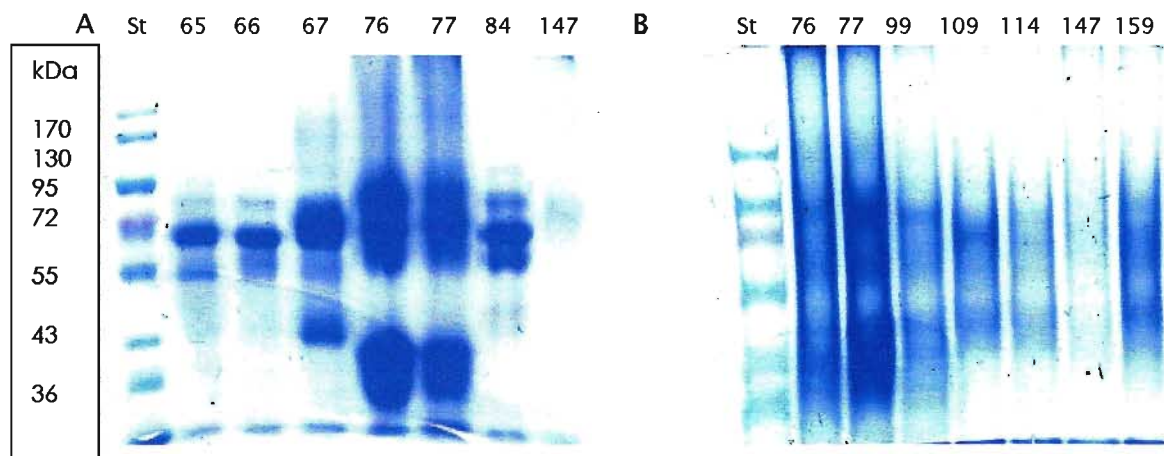


Fig 7A and B. Changes in protein bands appearance in honeys 76, 77, 99 and 114, during two months of storage. (A) Initial SDS PAGE, (B) two months of storage.

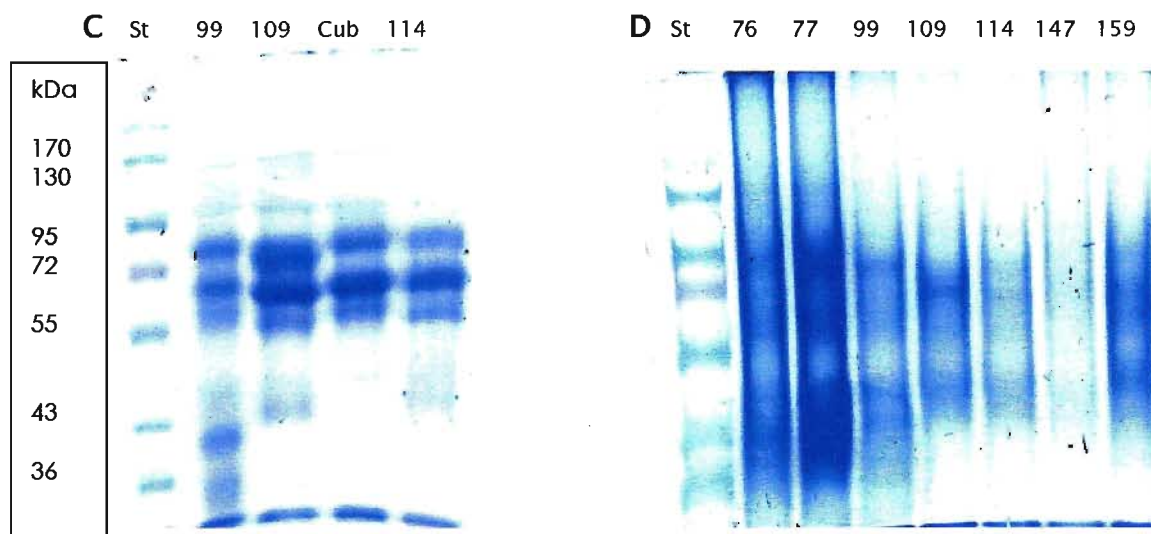


Fig 7C and D. Changes in protein bands appearances in honeys 109 and 99, during the two months storage. (C) Initial SDS PAGE and (D) after two months of storage

### 3.2.1 *Quantitation of changes in protein content in the SDS- gel bands in stored honeys*

The changes in protein concentration were quantified using Scion Image software. The protein content in honeys stored in different period of times (from March 2009 to Jan 2010) was analyzed, based on intensity of the band staining. The intensity was calculated from a number of dark pixels appearing in the designated area of a picture taken from a gel.

As an example, the changes in quantity of the 72 kDa of honey H77 was followed during 6 months of storage (Fig. 8).

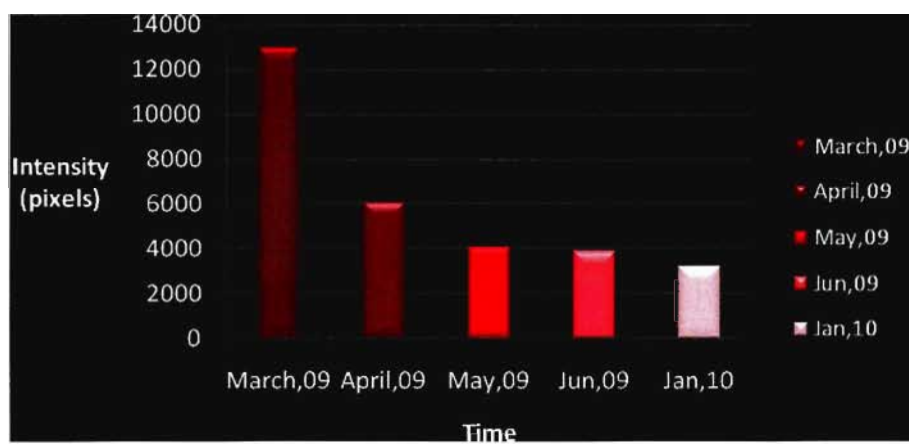


Fig 8. Comparison of the protein bands with storage time

It can be seen from Fig 8 that the change of intensity of the band was faster during the first three months of storage. After that time, the decrease in intensity reached a plateau.

This indicates that the storage of honey for prolonged time caused the reduction of the protein concentration, as evidenced by SDS- PAGE and the reduction occurred rapidly in the first three months of storage.



### 3.2.2 Proteolytic degradation

To test whether the marked reduction of protein concentration was due to proteolytic activity in our honeys, the following experiment was designed. Honeys H178 and H147 were incubated with bovine serum albumin (BSA) of known concentration for 15, 30 minutes and 1 hour, and the mixture was analyzed for the presence of BSA degradation products using SDS-PAGE.

By visual assessment, the concentration of BSA did not change in these experiments and no degradation products of lower molecular weights were detected in the gels (Fig. 9A and B). These figures represent the mixture of BSA and H178 and mixture of BSA and manuka honey H147 incubated for 15, 30 and 60 minutes

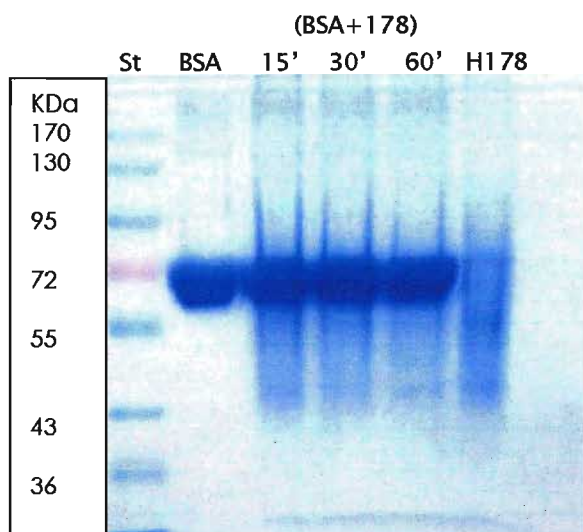


Fig 9A. Mixture of BSA and H178.

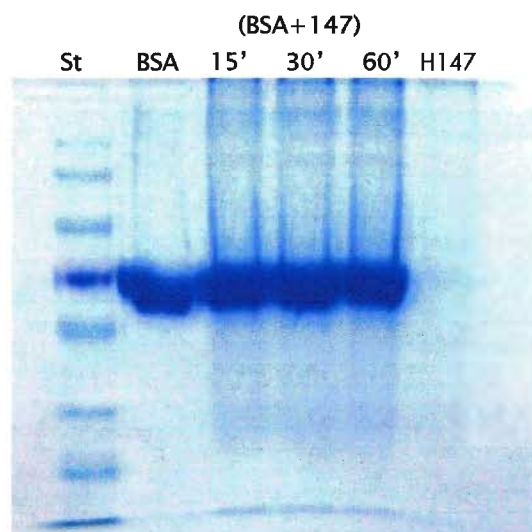


Fig 9B. Mixture of BSA and manuka (147).

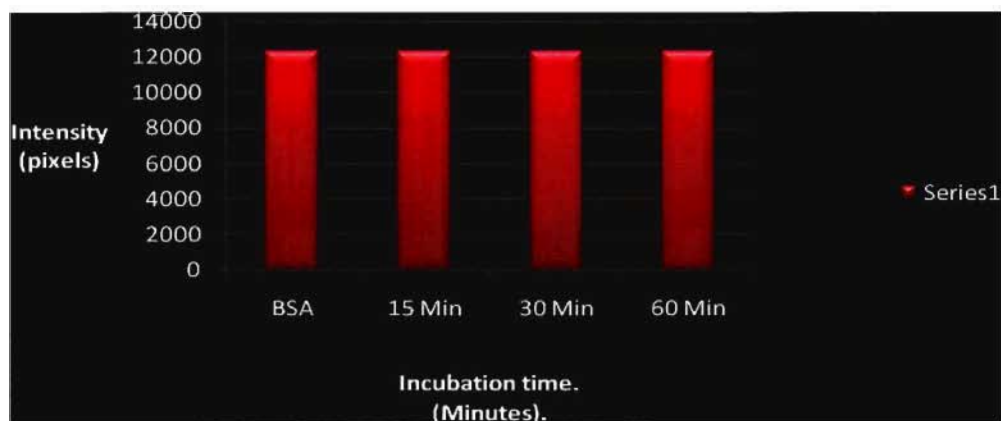


Fig 9C. Comparison of protein bands in a mixture of BSA+H178, incubated during 15, 30 and 60 minutes

To support these observations, the intensity of BSA bands at different points of incubation was analyzed using Scion Imaging system. As shown in Fig. 9C, the intensity of band representing the control ("BSA" bar) was the same as the intensities of BSA bands incubated with honeys for 15, 30 and 60 minutes.

These results were interpreted to suggest that honeys did not possess an internal proteolytic activity that could be responsible for the reduction of protein content.

### ***3.2.3 Protein complexation with polyphenols***

It has been hypothesized that a marked decrease in sharpness or definition of the protein bands may result from the protein modifications. The commonly observed protein modifications include protein glycosylation or complexation with polyphenols.

The protein-polyphenol complexation could explain the diffuse appearance of bands in SDS-PAGE, background staining and disappearance of bands. To test this possibility, ferric chloride was used to detect polyphenols in protein fractions, obtained by precipitation with ethanol. Fig 10.



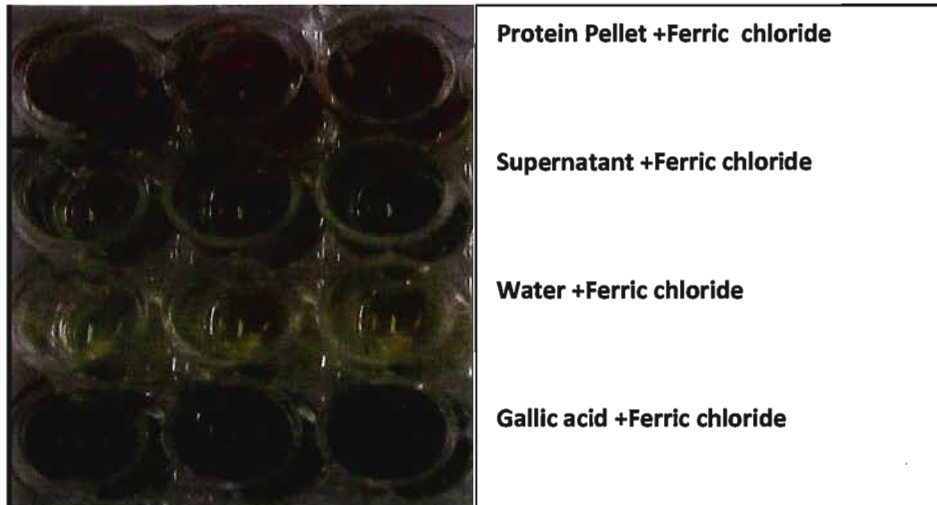


Fig 10. Color change after addition of ferric chloride

Gallic acid was used as a positive control while distilled water served as negative control. The simple experiment showed a positive reaction of the protein fraction with  $\text{FeCl}_3$  (formation of brown to greenish color) suggesting the presence of polyphenols.

## PART II

### *3.3 Separation of polyphenols from polyphenol–protein complexes*

The specific aim of Part II of the thesis was to design a methodology of protein purification from polyphenols and to determine quantitatively the concentrations of polyphenols and proteins.

#### *3.3.1 Protein profile of honeys H177, H178 and H179*

The second part of the project was conducted on three, freshly obtained honeys; buckwheat honey H177, blueberry honey H178 and wild flower honey H179.

The protein profiles of honeys H177, H178 and H179, the number of protein bands and their molecular size is shown in Table 10. As expected, buckwheat honey H177 showed a long protein pattern consisting of four protein bands, including a 38kDa band which has not been seen in other honeys. The three honeys shared the polypeptide bands of 82, 58 and 46kDa. (Fig 11).

Table 10. Molecular sizes (kDa) of polypeptide bands in honeys H177, H178 and H179

H177 (buckwheat)	H178 (blueberry)	H179 (wild flowers)
82	82	82
58	58	58
46	46	46
38	---	---

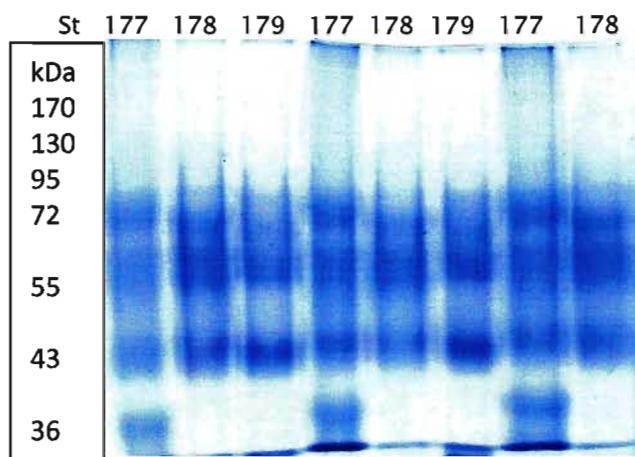


Fig 11. Protein profile of honeys H177, H178 and H179

### *3.3.2 Methods of polyphenol removal from protein-polyphenol complexes*

In order to separate polyphenols from polyphenol-protein complexes, two protocols were analyzed and their efficiency in polyphenol removal were compared.

Protocol 1(P1) consisted of precipitating proteins from honey samples using 95% ethanol. The protein precipitate was removed by centrifugation and the supernatant was saved to measure the total phenolic content using the Folin- Ciocalteau method. The proteins in the pellet were dissolved in water and analyzed, both quantitatively and qualitatively using a Pierce Protein Kit and by SDS- PAGE, respectively.

In Protocol 2 (P2), honey samples were treated with a mixture of equal parts of three alcohols; ethanol 80%, methanol 80% and propanol 70%. The samples were left at 4°C for overnight precipitation and after centrifugation the polyphenolic content was assessed in supernatant. The proteins in the pellet were analyzed both quantitatively and qualitatively using Pierce Protein Kit and SDS- PAGE, respectively.

The phenolics removal from the protein sample was monitored at two-and four-fold dilutions. In addition, at this stage the Silver Stain was used instead of Coomassie Blue to discern more details in the changes in protein concentrations.

Fig 12A shows the protein profile for honey H177 before phenolic extraction. Fig 7B shows the protein profiles after extraction of honey with either ethanol (protocol P1) or a mixture of three alcohols (protocol P2). In each case, 30  $\mu$ l of sample was loaded on the gel.

The results of SDS-PAGE demonstrated that the efficiency of phenolics removal from protein-phenolic complexes using both protocols was comparable. However, the protocol P1 caused the removal of one polypeptide band in honey H177. By applying Silver Stain instead of Coomassie Blue, it was possible to detect two more protein bands of a high molecular weight; 165kDa and 134kDa.

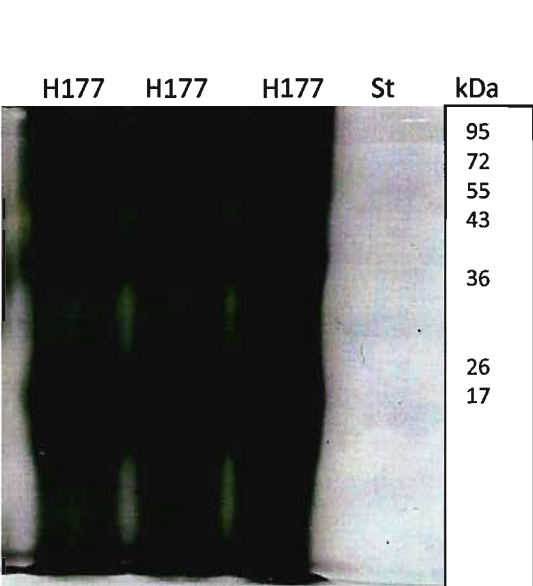


Fig 12A. Honey H177 before P1 and P2

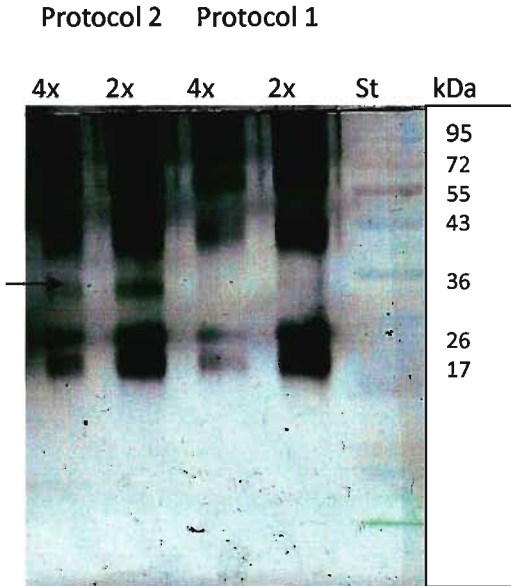


Fig 12B. Honey H177 after P1 and P2. The black arrow represents the missing polypeptide band applying P1.

Similar results as to the efficiency of polyphenol removal using these two protocols were obtained for light-color honey, wildflower H179 (Fig. 12C and Fig. 12D). While both methods removed some of the phenolics from protein-phenolic complexes, the

procedures only slightly improved the sharpness of the bands on SDS gels and the background staining, specifically in polyphenol-rich honey, such as buckwheat honey.

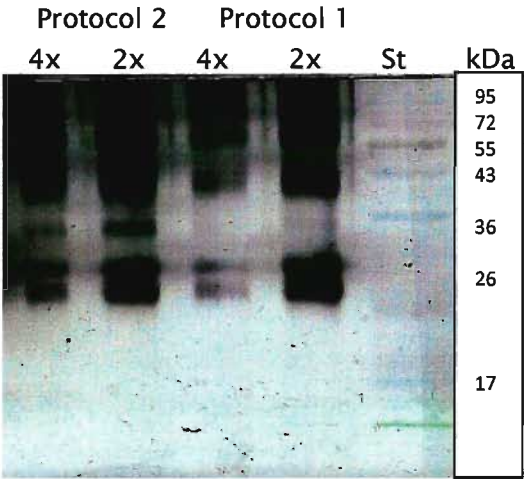


Fig 12C. Dark honey (Buckwheat) H177 treated with protocols P1 and P2.

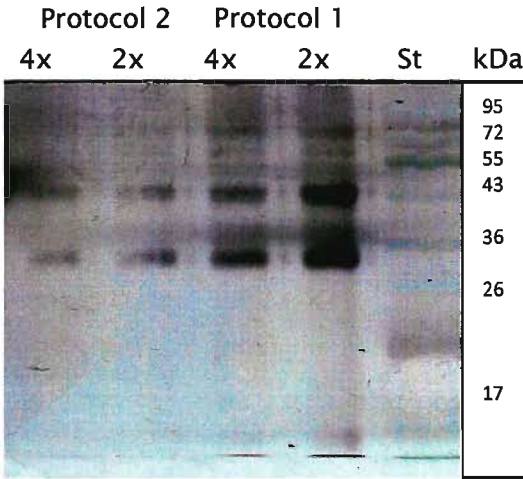


Fig 12D. Light honey (wild flower) H179 treated with P1 and P2.

### 3.3.3 Determination of polyphenolic content

To determine the total phenolic content in honeys and honey fractions obtained after treatment with protocols P1 and P2, the Folin –Ciocalteu method was employed. Honey solutions (25% v/v) were first purified using solid-phase extraction method (SPE) to remove impurities, which could interfere with the analysis, specifically reducing monosaccharides such as glucose and fructose. The total phenolic concentration was determined based on the standard curve, generated using a serial dilution of Gallic acid (1mg/ml) (Fig. 13).

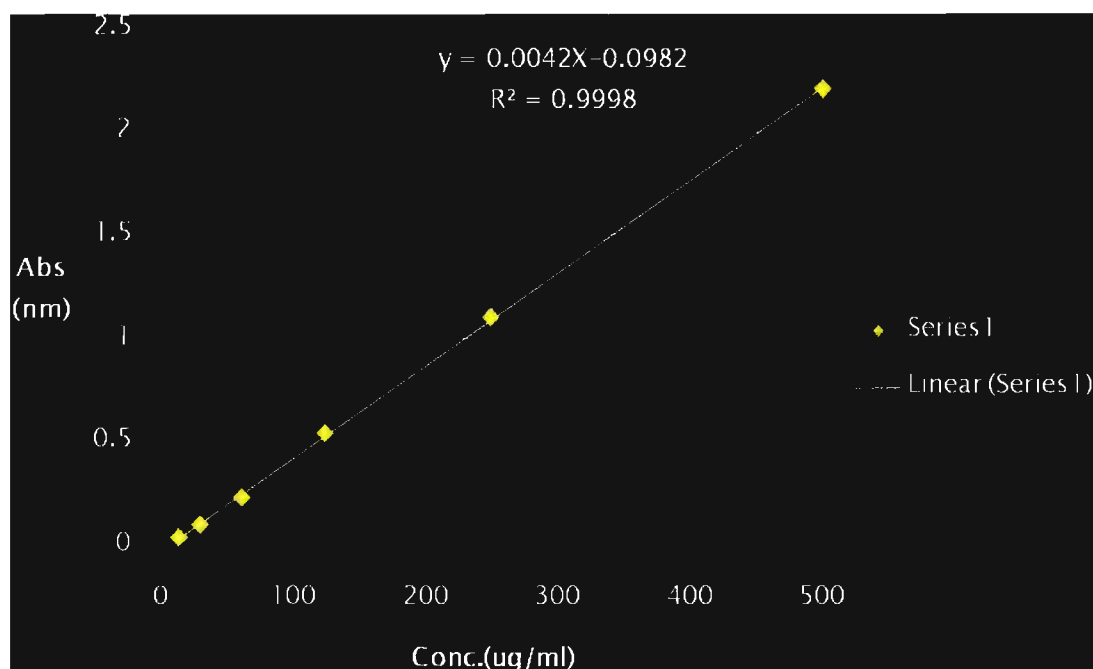


Fig 13. Standard Curve constructed using gallic acid as a standard to determine the phenolic content in honeys

The total phenolic content in buckwheat –H177, blueberry– H178 and wildflower –H179 honeys was determined before the phenolic removal. The results are summarized in Table 11.

Table 11. Color and total phenolic content in honeys H177, H178 and H179

Honey	Intensity of Color Net Absorbance (A <sub>560-720 nm</sub> )	Total phenolic content in honey (µg GAE/ml honey)	Total phenolic content in SN (µg GAE/ml honey)	
			P1	P2
Buckwheat H177	0.660	396.0 ± 17.3	144.8	160.0
Blueberry H178	0.231	163 ± 12.4	84.0	88.6
Wildflower H179	0.183	138.6 ± 11.9	74.0	78.4

Honeys were subjected to treatment with P1 and P2. The total phenolic content was measured in supernatants (SN) obtained after centrifugation and removal of protein pellet. The efficiency of polyphenols removal with alcohols of different polarity is presented in Fig. 14.

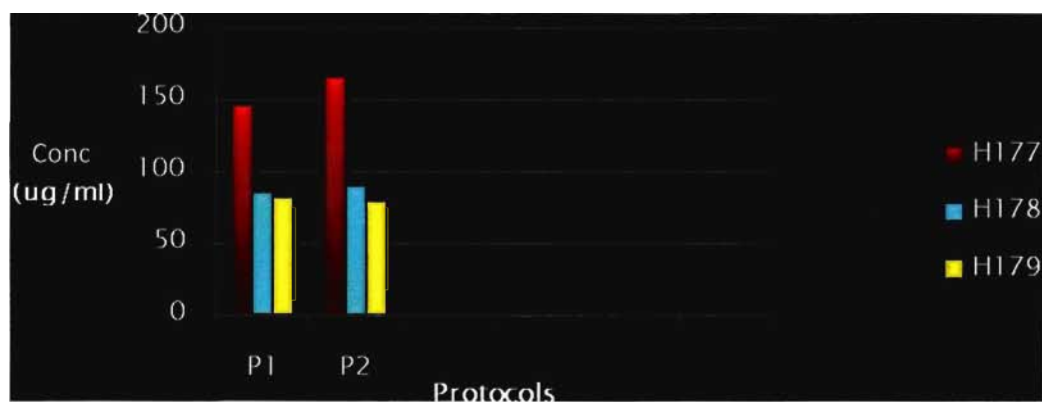


Fig. 14. Concentrations of phenolics in supernatants after treatment of honeys with protocols P1 and P2

As can be observed (Table 11 and Fig 14), approximately 40% to 56% of the polyphenols were removed from honey samples by extraction with either ethanol alone (P1) or three alcohols (P2). These results suggest that 60% to 44% of polyphenols were still bound to protein fraction (precipitate).

#### ***3.3.4 Determination of protein concentration in honeys***

The quantification of protein concentration was performed using Pierce BCA Protein Assay Kit. The standard curve was established using BSA as a protein standard of known concentration (1mg/ml) (Fig 15).

Honey solutions (50% v/v) were extracted and precipitated with either 80% ethanol (P1) or mixture of three alcohols (P2) and the protein concentration in precipitates was established after dissolving precipitates in distilled water.

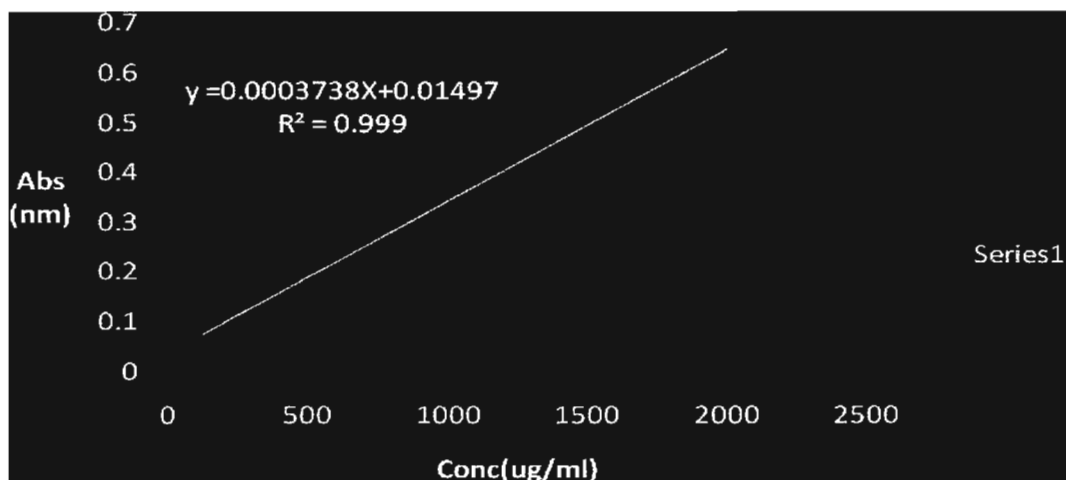


Fig. 15. Protein concentration standard curve constructed on known concentrations of BSA

The results indicated that protein concentrations in the precipitates after honey extraction using protocols P1 and P2 were very similar (Fig. 16). Extraction of honey with a mixture of three alcohols (P2) was slightly more efficient and produced higher recovery of proteins.

It became evident that buckwheat honey H177 contained the highest amount of protein, 1465ug/ml. Blueberry honeys H178 and 179 contained 212 and 184ug/ml of protein (respectively). (Fig.16). These were the values of protein concentration determined when the honeys arrived to the laboratory.



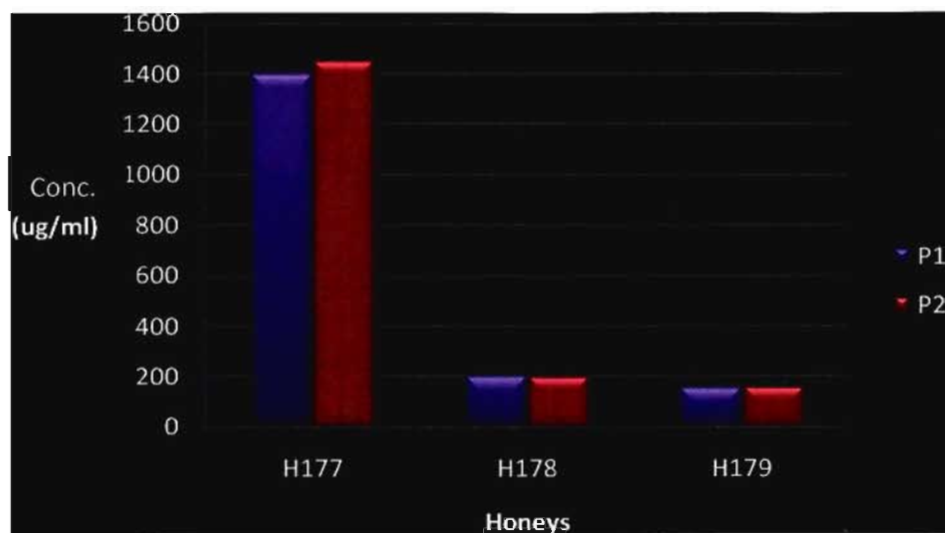


Fig 16. Protein concentration determined in H177, H178 and H179, applying P1 and P2

### 3.3.5 . Determination of the protein concentration in honeys using

#### *Pierce BCA Protein Assay Kit*

To determine a protein concentration, honeys were extracted/precipitated with a mixture of three alcohols (protocol P2) and protein concentration in precipitates was assessed with a Pierce BCA Protein Assay Kit.

Table 12 shows the protein concentration for ten honeys from different geographical regions and different botanical sources. Values of protein content ranged from 96.2 to 3344.6 $\mu$ g/ml. Light-color honeys possess the lowest protein concentrations (wildflower H179, blueberry H178). The highest protein concentration was found in the darkest honeys (buckwheat honeys H125, H77 and H76). These results were in good agreement with our data from SDS-PAGE, where light color honeys showed fewer protein bands and the bands showed low intensity of staining.

Table 12. Protein concentrations of honeys

Honeys	Protein Conc. ( $\mu\text{g/ml}$ )	Honey color (Net absorbance) (au)
H177	623	0.66
H178	156.5	0.165
H179	96.2	0.131
H76	1455.8	0.965
H77	1941.5	1.886
H99	852.2	0.298
H109	616.3	0.151
H114	583.9	0.172
H125	3344.6	1.880
H147 (Manuka)	321	0.539

### *3.3.6 Relationship between the protein concentration in honeys and the honey color*

An interesting relationship was observed between the intensity of color of honey (net absorbance) measured at  $A_{(560-720 \text{ nm})}$  and its protein content. Dark-colored buckwheat honeys (H125, H77, H76 and H177), were the richest source of proteins (3344.6, 1941.5, 1455.8 and 623  $\mu\text{g/ml}$ ), respectively and they showed high net absorbance values (Table 13). Light-color honeys showed the lowest protein concentrations and low net absorbance values (Table 13). Pearson  $r$  correlation indicated

that there was an extremely significant correlation between honey color and its protein concentration ( $R=0.976$ ,  $p < 0.0004$ ).

This result suggest that protein concentration contribute to the final honey color.

### ***3.3.7 Effect of polyphenol–protein interaction on protein bands***

#### ***detection on SDS–gels***

Along with the rest of the honeys tested in this project, Manuka honey (H147) was assessed as a reference honey. According to the literature, Manuka is known for a low or almost absent protein content. Surprisingly, in my study, Manuka honey H147 showed a relative high protein concentration of  $321\mu\text{g/ml}$ . This value indicated protein content higher than some of our light honeys such as H178 and H179. This apparent discrepancy was further investigated by extracting Manuka honey with a mixture of three alcohols (protocol 2) in order to remove phenolics and analyzing its protein profile. Fig 17A and 17B show the protein profiles of Manuka honey H147 before and after removal of phenolics with protocol 2. Before treatment, the polypeptide bands were barely visible and appeared as a very broad diffuse band, however after polyphenol removal two clearly defined bands were observed.

These results gave additional strong support to our notion that proteins in honey are complexed with polyphenols. The binding of polyphenols to proteins mask their recognition by commonly used stains such as Coomassie or Silver stain on SDS gels. It can be postulated that depending on the degree of the complexation, the protein bands will either appear as series of closely located bands, giving the impression of fuzziness, or will be completely blocked by polyphenols, preventing the binding of the stains.

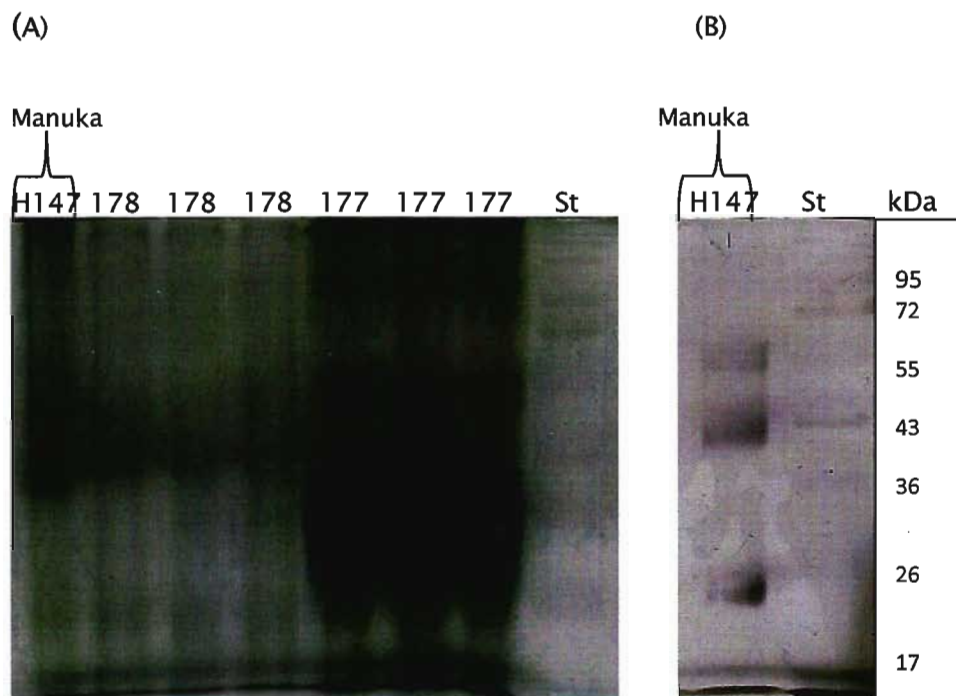


Fig 17. Protein profiles before (A) and after (B) extraction with a mixture of three alcohols. Honeys used are H177, H178 and Manuka H 147.

## **PART III**

### ***3.4 Effect of storage time and temperature on the protein and phenolic concentrations in honeys***

It is known that honey stored for prolonged periods of time loses some qualities such as color, aroma, as well as, some biological functions, such as antibacterial and antioxidant activity. The reason for the deterioration of honey quality with time has not been sufficiently investigated. The specific aim of Part III of the project was to investigate the effect of honey storage on the protein and polyphenol concentrations.

#### ***3.4.1. Storage time and protein concentration in honeys***

The effect of storage conditions on honey proteins and polyphenols was investigated on three honeys. Buckwheat honey H177, blueberry honey H178 and wildflower honey H179 were divided into portions at the day of their arrival to the laboratory. Each portion was stored separately at three temperatures: room temperature (RT), -4 °C and -20 °C. The samples of stored honeys were analyzed once a month for a period of 6 months for a protein concentration, protein profile and phenolic content.

Fig 18 shows changes in the protein concentration of honeys H177, H178 and H179 stored at room temperature from December, 2009 until March, 2010. The initial protein concentration in these honeys was 1465.7, 212 and 184 µg/ml for buckwheat, blueberry and wildflower, respectively. However, upon storage at room temperature, honeys showed a rapid decrease in protein concentration during the first three months of storage. The most visible decrease was observed for honey H177 and amounted to the loss of 60% of proteins.

In all honeys, in the last two months of storage the protein concentration reached a plateau, in which practically the values remained stable.

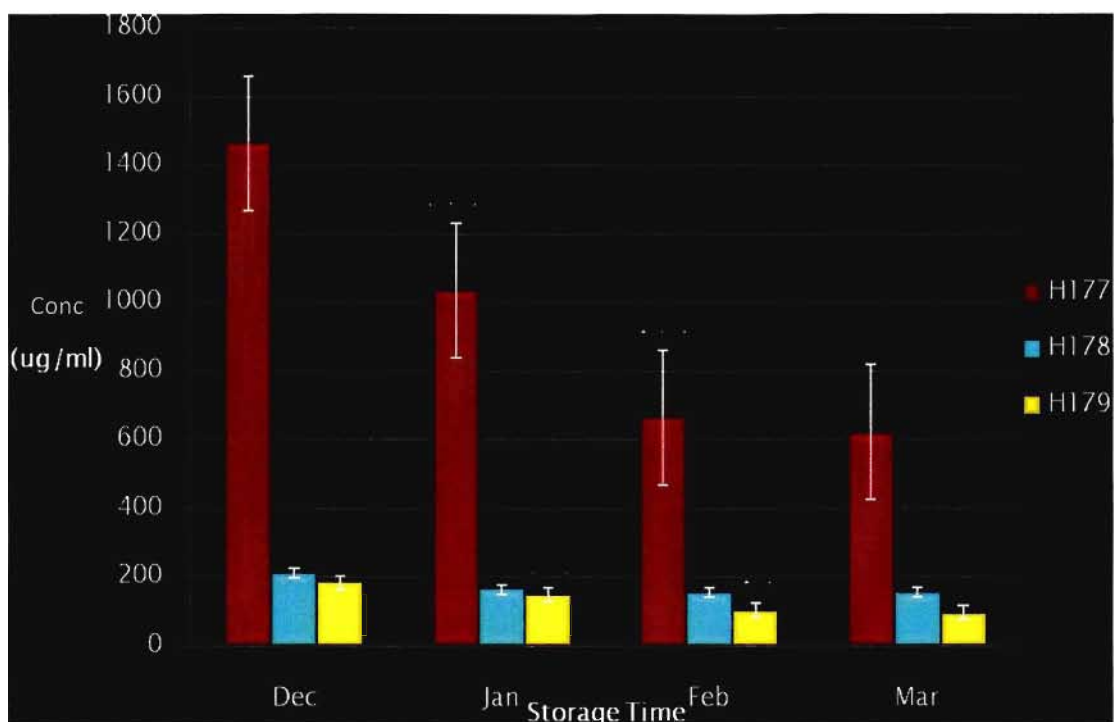


Fig18. Comparison of protein concentration in honeys H177, H178 and H179, stored for six months, at room temperature.(n=9).

The statistical analysis showed a significant reduction of the protein concentration in honeys stored for 6 months at room temperature (ANOVA,  $p < 0.001$ ).

#### ***3.4.2 The effect of storage temperature on the protein concentration***

##### ***in honeys***

The effect of storage temperature on the levels of proteins was investigated in honeys H177 (darkest) and H179 (lightest). Honeys were stored at three temperatures: at room temperature, 4°C and -20°C. Protein concentrations in these honeys were measured every month for 6 months.

Independently of temperature at which honeys were stored, the decrease in protein content was observed with time of storage (Fig. 19, 20, and 21). Therefore, storage time

was the most critical factor for the reduction of protein content in honeys. The rapid reduction was observed in the first two months of storage, independently of storage temperature (ANOVA,  $p < 0.0001$ ). No significant differences in protein contents were found between second and third months of storage (ANOVA,  $p > 0.05$ ) (Fig 19, 20 and 21).

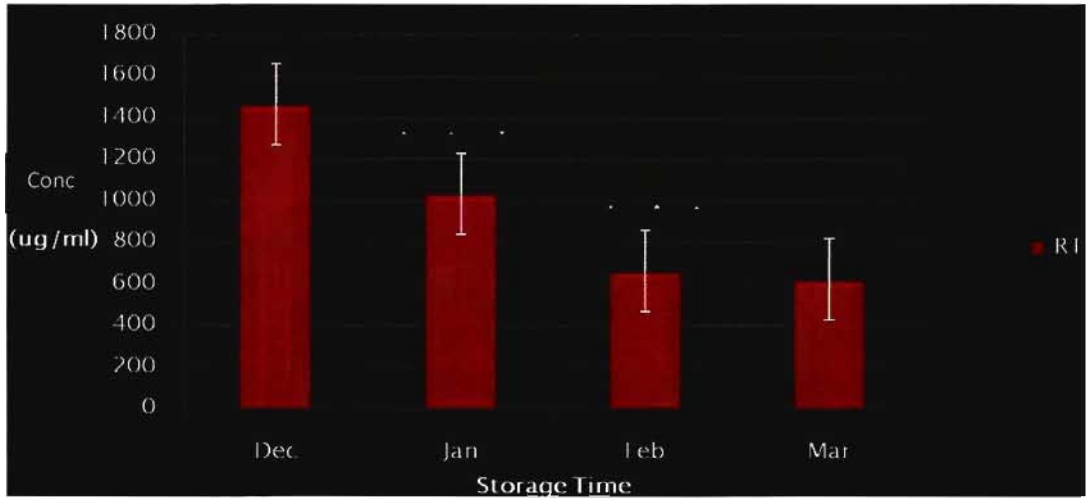


Fig 19. Changes in protein concentration for honey H177 at RT.(n=3).

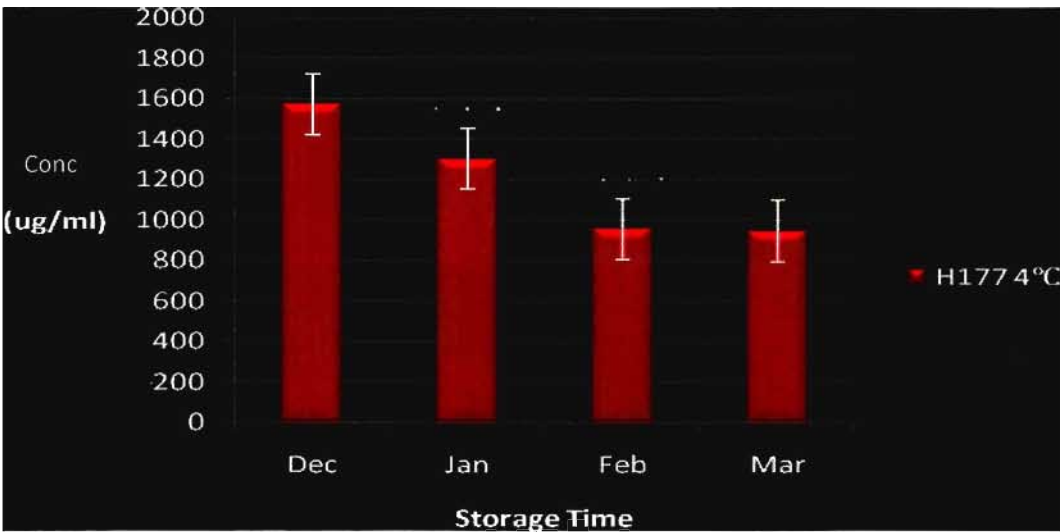


Fig 20. Changes in protein concentration for honey H177 at 4°C.(n=3).

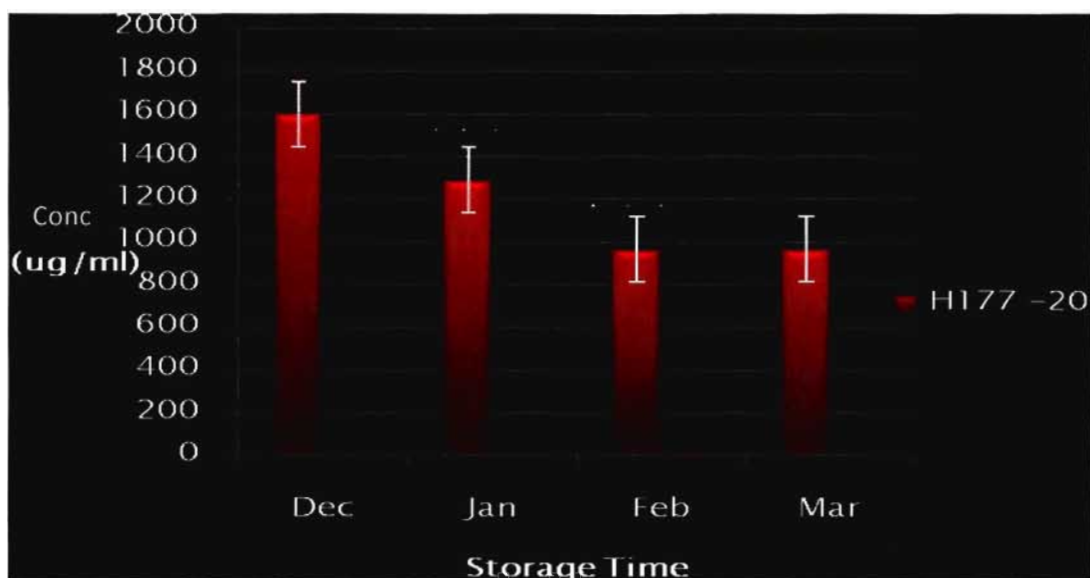


Fig 21. Changes in protein concentration for honey H177 at  $-20^{\circ}\text{C}$ . (n=3).

While the reduction of protein content was observed at all storage temperatures, the samples of honeys kept at  $4^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  showed slower rates of decrease (Fig. 19, 20 and 21), indicating that protein concentrations were better preserved at colder temperatures.

The degree of protein loss was dependent on the botanical origin of honeys or rather the initial total protein concentration. Analyzing the results of protein concentration in buckwheat honey H177 versus wildflower honey H179, a larger loss of protein was observed in the darker honey H77 than in lighter honey H179 (Fig. 18).

The decrease in protein content during storage of light-color wild flower honey H179 was also observed. As in the case of honey H177, there was a significant reduction of the protein content in honey H179 in the first two months of storage (ANOVA,  $p < 0.0001$ ) (Fig 22, 23 and 24). No differences in protein concentrations were found between the second and third month of storage (ANOVA,  $p > 0.05$ ).



Similarly to honey H177, the colder the temperature during storage, the better preservation of protein content in honey H179. (Fig. 22, 23, and 24).

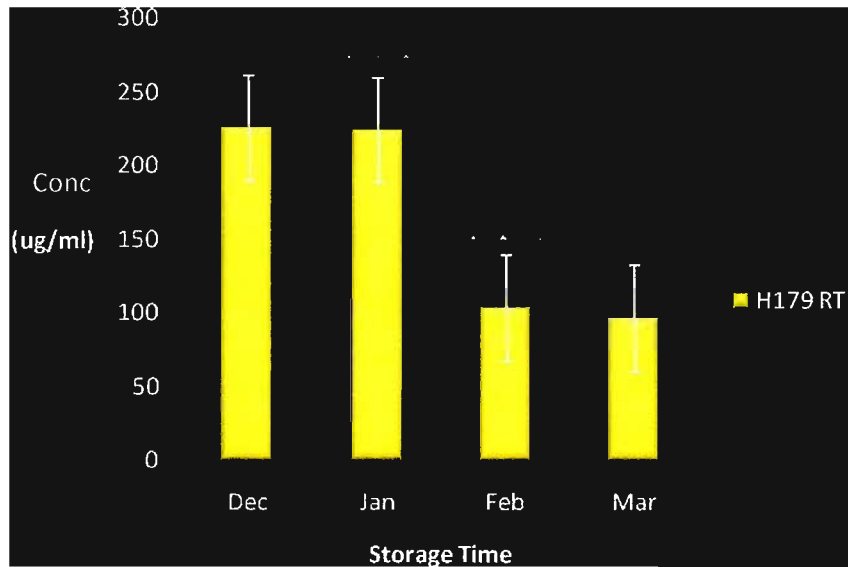


Fig 22. Changes in protein concentration for honey H179 at RT with storage time.(n=3).

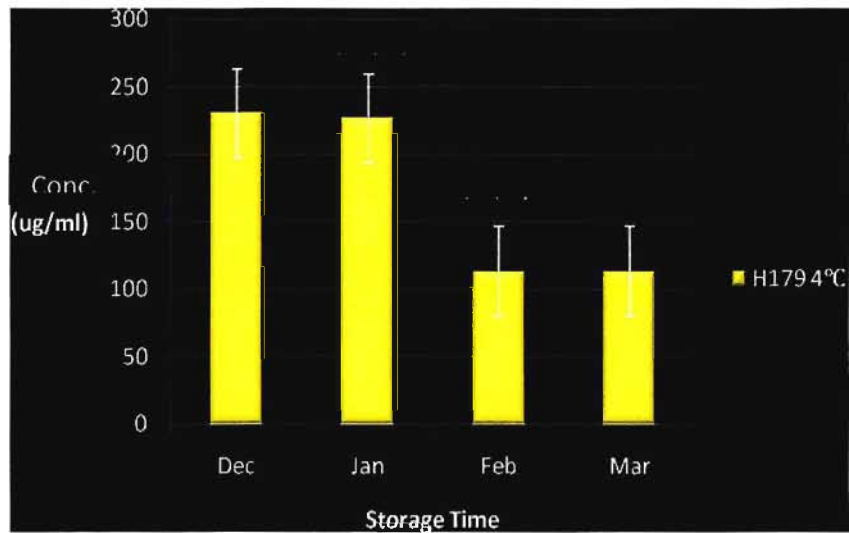


Fig 23. Changes in protein concentration in H179 at 4°C with storage time . (n=3).

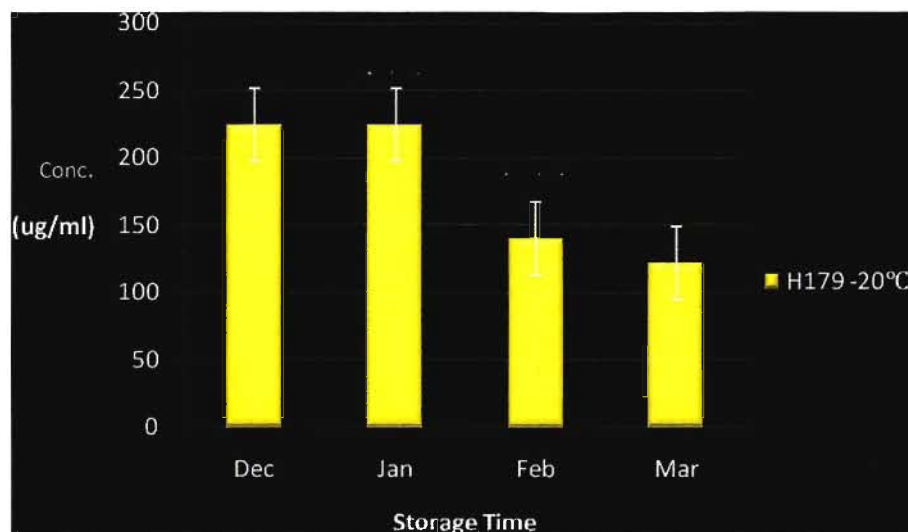


Fig 24. Changes in protein concentration in H179 at -20°C.(n=3).

### ***3.4.3 Changes in phenolic content during honey storage***

In parallel to the determination of the protein content in precipitates after extraction of honeys with protocol 2 (P2), the supernatants were used to estimate the total phenolic content in the same honeys. The total phenolic content was measured once a month for a period of six months using Folin– Ciocalteau method. The results are presented in Fig 25.

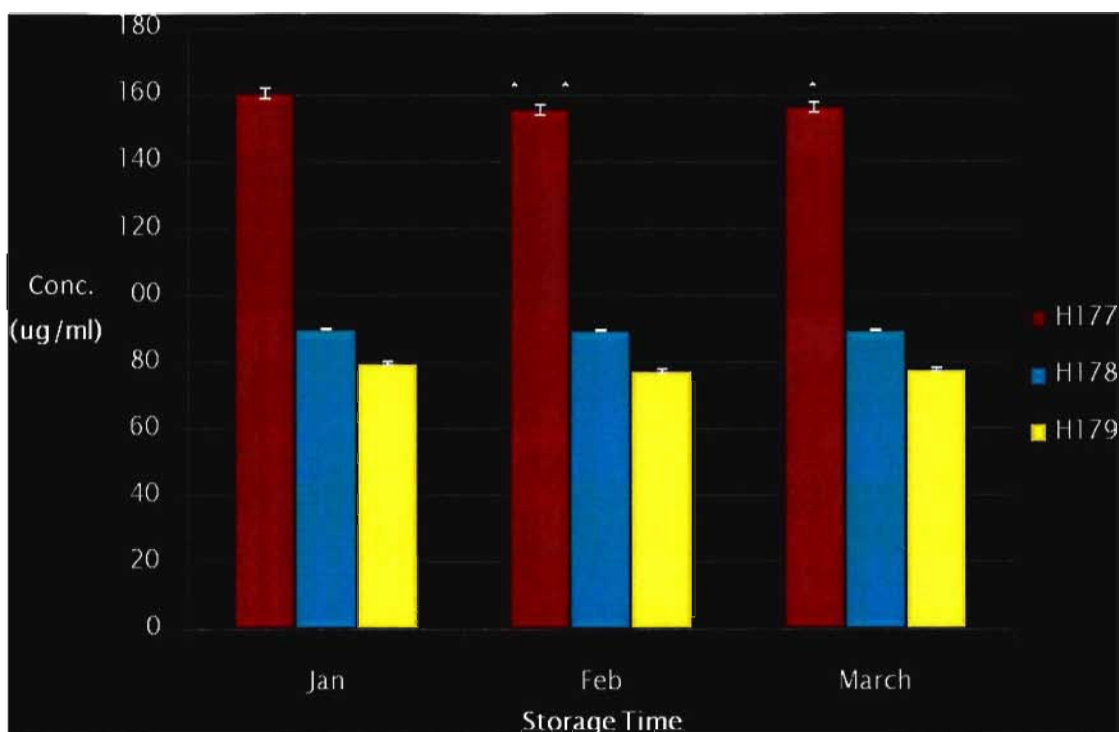


Fig 25. Free phenolic content in honeys H177, H178 and H179 with storage time.(n=9).

In contrast to the protein concentration that showed a remarkable decrease during six month storage of honeys at room temperature, the phenolic content in SNs was reduced only slightly. Despite that, the reduction in the phenolic concentration during three months of storage however was significant ( $p < 0.001$ , Jan to Feb and  $p < 0.05$  for Feb–March).

#### *3.4.4 Changes in phenolic content in honeys stored at different temperatures*

The temperature at which honeys were stored influenced their total phenolic content. There was a significant decrease in phenolic concentration in both honeys, H177 and H 179, stored at  $-20^{\circ}\text{C}$  versus room temperature (ANOVA,  $p < 0.001$ ) and between  $4^{\circ}\text{C}$  versus  $-20^{\circ}\text{C}$  (ANOVA,  $p < 0.01$ ). No significant changes were found

between the phenolic content in honey H177 stored at RT vs 4°C, ( $p>0.05$ ) (Fig. 26 and 27).

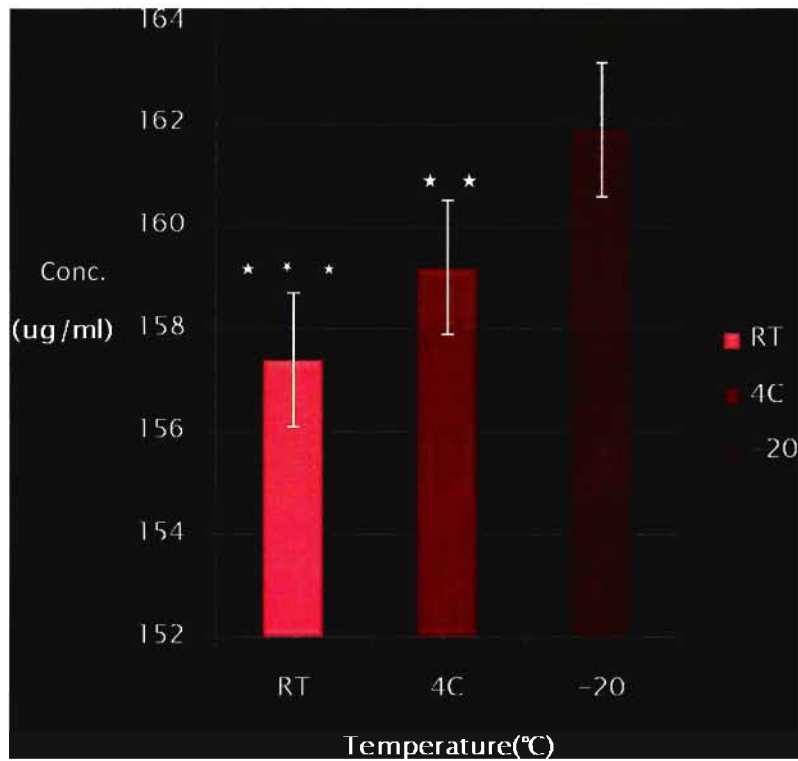


Fig 26. Total phenolic content in buckwheat honey H177 stored at different temperatures. (n=3).

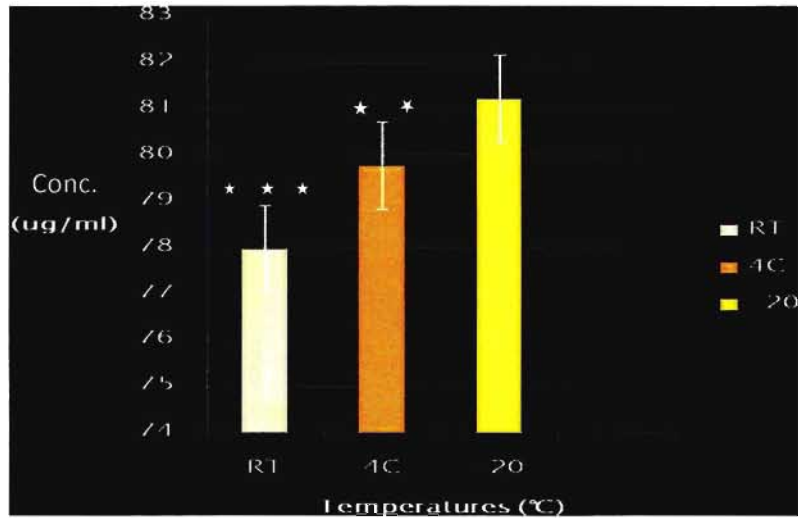


Fig 27. Total phenolic content in wildflower honey H179 stored at different temperatures. (n=9).

## PART IV

### 3.4.5 Liquid chromatography/ Mass spectrometry analysis of polyphenol

#### *removal from honey by liquid-liquid extraction*

The diagram below presents the protocol which was designed to monitor polyphenol removal from protein –polyphenol complexes. (Fig. 28).

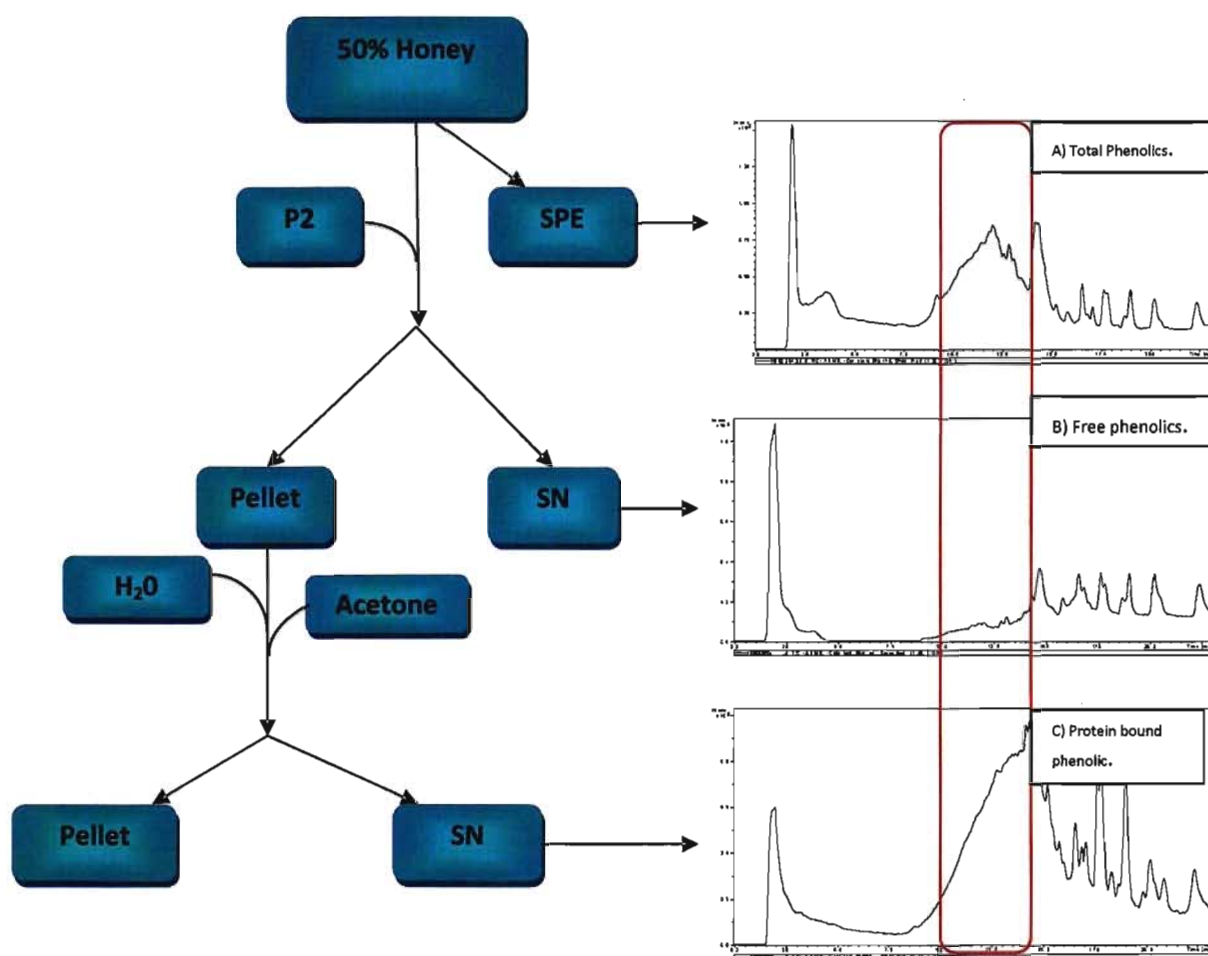


Fig 28. Polyphenol content in honey H177 and protein-bound polyphenols

One ml of honey H 177 solution ( 50% v/v) was purified through solid phase extraction (SPE) using Oasis cartridges as described in Materials and Methods. The eluent (1 ml) was lyophilized and reconstituted in 50 $\mu$ l of methanol. The 5 $\mu$ l of this

sample was injected into a Zorbax LC/MS column. Chromatogram A shows the total ion current (TIC) of all phenolic compounds present in honey.

The 50% honey solution was extracted with a mixture of three alcohols (protocol P2) and precipitate was separated from supernatant (SN) by centrifugation. The sample of supernatant was lyophilized and reconstituted in 50µl of methanol. 5µl of this sample was injected into the Zorbax LC/MS. Chromatogram B represents phenolic compounds which were not bound to proteins in honey as they were easily removed by extraction with three alcohols.

The pellet of proteins precipitated by a mixture of three alcohols, was dissolved in distilled water. The sample then was extracted with acetone as described in Materials and Methods. Acetone is a known solvent used in purification of protein extracts from residual contaminations with phenolics. Phenolics found in acetone extracts are those bound to the proteins. Chromatogram C (Fig 28) represents phenolics recovered from protein–polyphenol complexes.

Comparison of the three chromatograms A, B and C (Fig 28), indicate that a significant portion of total phenolics is bound to proteins (chromatogram C).

The total phenolic content was measured in the samples subjected to the LC/MS using the Folin–Ciocalteu method. As shown in Table 13 the majority of phenolics present in the whole honey was recovered in the acetone fraction. This suggests again that proteins and phenolics form complexes.

Table 13. Total phenolic content: free phenolics and phenolics removed from protein complexes

	H177 ( $\mu\text{g/ml}$ )	H178 ( $\mu\text{g/ml}$ )	H179 ( $\mu\text{g/ml}$ )
Full Honey 50% (SPE)	396	163	138.6
Protocol 2 (Free phenolics)	160	88.6	78.4
Treatment with Acetone (protein bound-phenolics)	236 (59.6%)	74.4 (45%)	60.2 (43.4%)

***3.4.6 LC/MS analysis of compounds present in full buckwheat honey  
and in fractions of unbound and protein-bound phenolics***

A comparison of the Total Ion Current (TIC) chromatograms revealed that the most visible changes in the composition of fractions obtained from the extractions of honey with organic solvents occurred at the retention times 10.0 to 14.00 minutes Fig. 29. The full honey was very rich in phenolic compounds Fig. 29A. The LC/MS analysis in negative mode showed a very complex mixture of compounds eluting at very close retention times to each other Table 14. Honey treatment with a mixture of three alcohols removed only a small portion of the compounds present in the 10–14 min peak of full honey (Fig.29B). The acetone extraction of the precipitated protein-rich fraction, released the largest portion of polyphenolic species (Fig. 29C). The retention times of the main peaks appearing in the TICs of full honey and in the fractions obtained with a three-alcohols and acetone extractions were compared. As shown in Table 14, acetone extraction of protein-rich fractions (see diagram Fig 28) removed very specific groups of polyphenolic compounds with retention times of 11 to 12 min, 12.9 min, and 13.5 to 13.7min, Table 14.

To shed some light on the nature of these compounds, a series of commercial polyphenolic acids and flavonoids were used as standards for LC/MS and run under the

same chromatographic conditions. Table 14 summarizes the information obtained from LC/MS of these standards. From these data, it appears that the acetone extraction specifically removed flavonoids from protein-polyphenol complexes, including Rutin, Quercetin and Apigenin as well as Cinnamic acid. Some flavonoids were present in full honey and in all fractions, such as Kaempferol, Pinobanksin and Luteolin.

Even though the identification of polyphenols was not an objective of this project, the comparison of RT and mass spectrum of these peaks with RT and mass spectrum of flavonoid standards allowed us tentative identification flavonoid aglycones which are typically present in honeys such as Apigenin ( $m/z$  269), Kaempferol ( $m/z$  285) and Myricetin ( $m/z$  315) (Fig. 30 and Fig. 31).



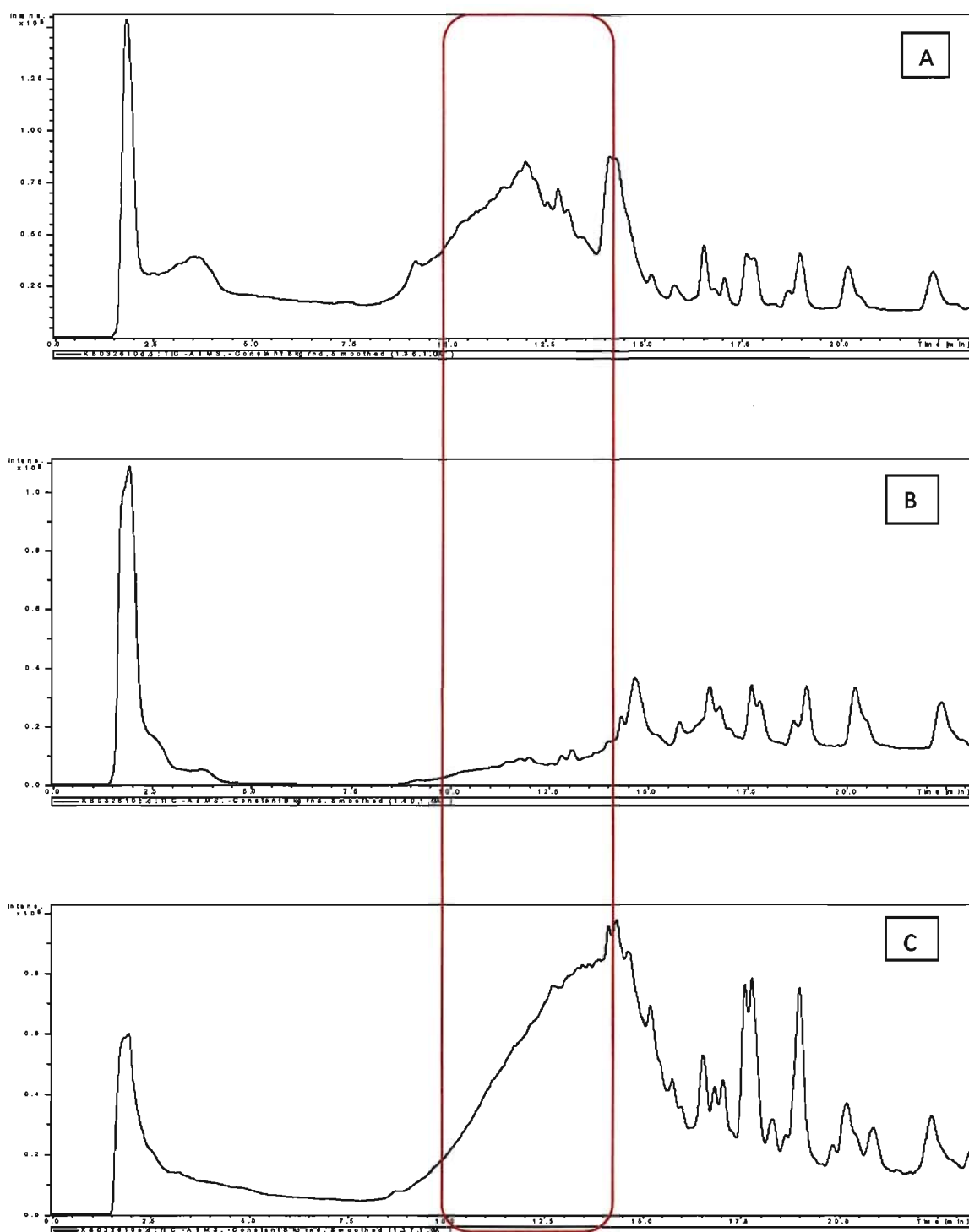


Fig 29. LC chromatogram of A) Total phenolic content after SPE. B) Free phenolics after protein precipitation from honey solution. C) Phenolics removed from protein complexes by acetone. Framed area correspond to RT 10.0 to 14.0 min

Table 14. Comparison of main peaks appearing at RT 10 to 14min in TICs of full honey and extracted fractions

Standard Names	Mass ion [M-H] <sup>-</sup> (m/z)	Polyphenolic Standards RT(min)	Full honey Main Peaks RT (min)	Unbound phenolics (SN) Main Peaks RT(min)	Bound phenolic (acetone fraction) Main Peaks RT(min)	Mass ion [M-H] <sup>-</sup> (m/z) Dominant peak
			10.054			
			10.518	10.603-10.748		
Myricetin	317		10.942	10.898	10.920	315
Cinnamic acid	147	11.1	11.178		11.021	
Rutin	609	8.0	11.543		11.551	
Quercetin gluc	463		11.775		11.736	
Quercetin	301	11.8	11.896		11.816	
			12.026	12.070	11.999	
			12.184	12.203	12.211	
			12.381	12.391		
Unidentified			12.641		12.509	
Luteolin	285	12.5	12.635	12.656	12.622	
			12.812	12.815		
Hesperitin	301	13.0	12.958		12.901	
Pinobanksin	271	13.15	13.189	13.159	13.159	271
			13.439			
Apigenin	269	13.2	13.531		13.530	269
			13.781		13.730	
			13.902	13.907	13.948	
Kaempferol	285	14.19	14.110	14.108	14.108	285
			14.245			
			14.498			

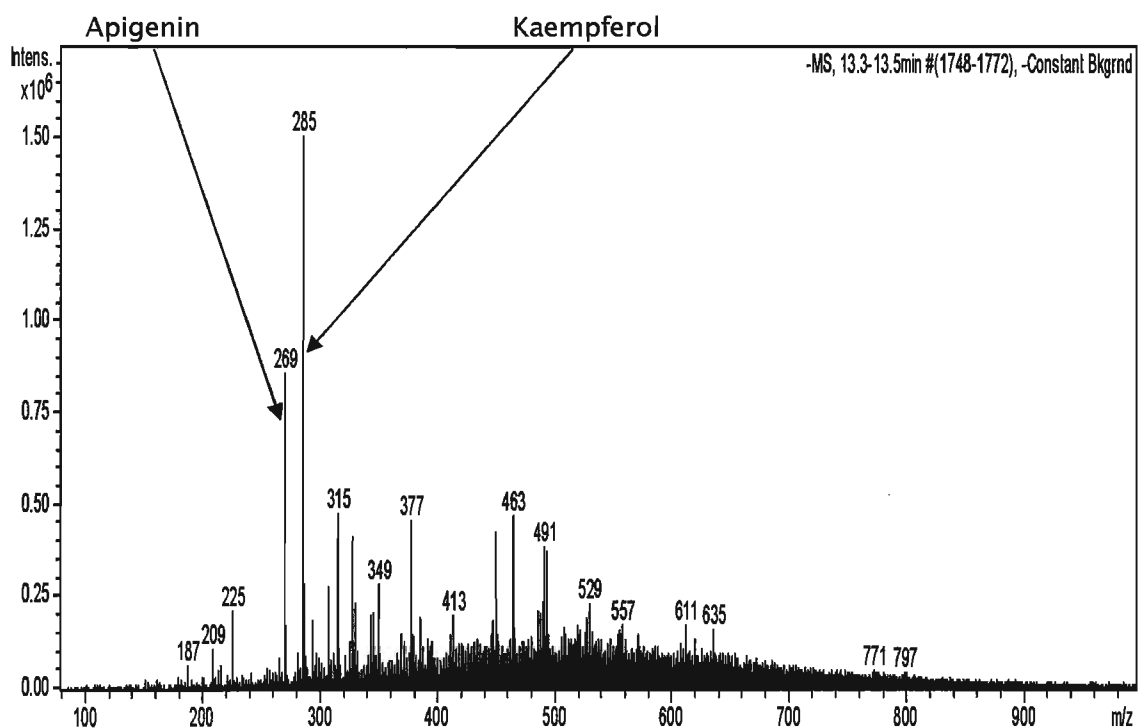


Fig 30. Mass spectrum of compounds eluted at RT 13.5 min. Comparison to the LC/MS of flavonoid standards allowed tentative identification as Apigenin, m/z 269, Kaempferol, m/z 285 and Myricetin, m/z 315 and their hexose (-162 amu) and rhamnose (146) derivatives

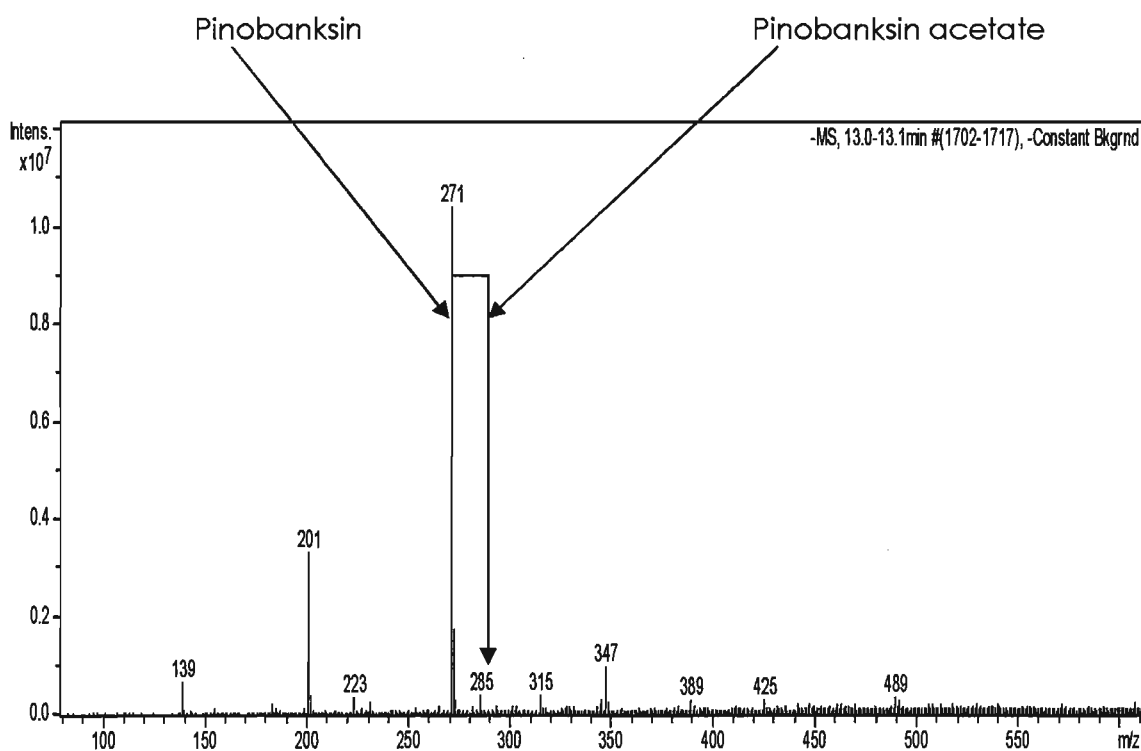


Fig 31. Mass spectrum of compounds eluted at RT 13 min., m/z 271 and m/z 285

It is well established that flavonoids in honey occur in a glycosylated form. The presence of hexose unit was identified from the appearance of 162 mass units, which correspond to the loss of a hexose such as glucose (Fig. 32).

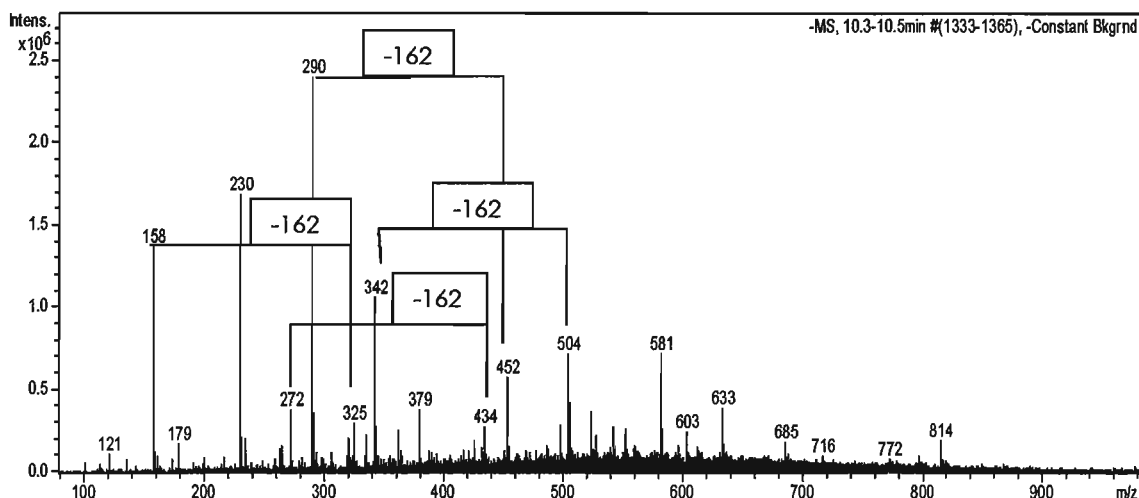


Fig 32. Mass spectra of compounds eluted at retention time 10.5 min Mass ion of 162 amu

By applying the combination of the extraction of honey with solvents of decreasing polarity and the LC/MS analysis of these fractions, it was possible to identify flavonoids as main polyphenols involved in the protein–polyphenols interaction. These polyphenols included cinnamic acid as well. To my knowledge, this is the first time that flavonoids are recognized as main polyphenols involved in protein complexations in honey. In addition, LC/MS analysis documented that flavonoids involved in protein binding are glycosylated. The protein binding by flavonoids could be disrupted by acetone, that could suggested that the binding is of a strong nature.

## 4. Discussion

Honey has a positive image in general public as a food product and a food with some health benefits. The image has been created based on a long history of usage of honey as a natural sweetener and traditional medicinal product Crane, (1990). The recent interest in natural and healthy food initiated intense research into honey components responsible for its health-stimulating properties.

Chemical components in honey originate from nectars collected by honey bees. The variations in the honey composition are related to botanical sources of nectar. In addition to the constituents originated from plants, called phytochemicals, honey is enriched with compounds which originate from secretion of hypopharyngeal glands of bees. Those of honey components originated from bees are shared between honeys. In addition, nectar carbohydrates, that are a source of honey sugars, comprise 80% of the dry mass of all honeys. In this context, the compounds that differentiate one honey from the other are compounds which originate from plants. For this reason, most of the research on the “therapeutic” components of honeys is directed to phytochemicals, mostly polyphenols. Relatively less interest was devoted to the proteins, their types, concentrations, profiles and their role in honey.

The objective of my thesis was to investigate honey proteins, their profiles and concentrations in different honeys. During the study, however, several unexpected observations were made that re-directed my interest toward the protein-polyphenol interactions and complexation. This involved different methods of removing protein from polyphenol complexes, the analysis of stability of proteins and polyphenols during honey storage and a potential type of chemical binding between proteins and polyphenols.

### ***General characterization of honeys used in the project***

Honeys, derived from different floral sources, are usually classified based on their color. Honeys showed very different colors, varying from dark brown to almost white. The color of honeys used in this project was estimated based on spectrophotometric method by measuring a net absorbance ( $A_{560-720\text{ nm}}$ ). Among thirteen honeys analyzed the color ranged from 1.88 absorbance unit (au) of dark buckwheat honey to 0.085 au of light-color pumpkin honey. Honey color is the result of compounds that absorb light in visible range, called chromophores. Main chromophores of honey are polyphenols, carotenoids and terpenes. Many researchers found that honeys with dark color have a higher amount of phenolic compounds (Ghedof and Engeseth 2002; Meda et al., 2005). This compilation of literature data became an important lead in several parts of my study.

Another general characteristic of honey is the pH. In honeys used in this project, the pH ranged from 3.59 to 4.83. Lighter honeys showed more alkaline values of pH while darker honeys showed more acidic values. The acidic pH in honeys depends on the amount of gluconic acid that is produced by the enzyme glucose oxidase during glucose oxidation (White et al., 1963). During the reaction, hydrogen peroxide is formed as a by-product. Studies indicated that gluconic acid, in equilibrium with gluconolactone, is the principal honey acid (Stinson et al., 1960). When hydrogen peroxide accumulates, it inactivates the enzyme and causes the breakdown of D-gluconolactone to gluconic acid. Accumulation of gluconic acid reduces pH of the solution (Kleppet, 1966).

Other compounds include non-aromatic and aromatic acids (Huidobro and Simal, 1984, Bogdanov, 2010), respectively. It has been also suggested that phenolic acids found in a larger amount in darker honeys might contribute to the acidity.

In this project, the acidity of honeys ranged from pH 3.59 to 4.83 and these values were in good agreement with literature data reported by Malika, Mohamed and Chakib (2005) and the National Honey Board (1996).

Therefore, the color and pH of honeys used in this study were within values reported in the literature.

### ***Proteins in honey***

The proteins of honey are largely unknown because they are only minor constituents of honey comprising of 0.5 to 1% of the honey dry mass (Saravana and Mandal, 2009).

The initial purpose of the project was to investigate the protein profiles in different honeys. With this in mind, the protein profiles were analyzed in 13 honeys using SDS-PAGE. In the SDS-PAGE analysis of honeys, a total of eight protein bands were observed but the number of polypeptides bands in different honeys varied from 1 to 8. Four bands in the protein profile were common for all tested honeys (with the exception of Manuka honey) and included polypeptides of 82, 72, 60 and 52kDa. It was expected to find some polypeptides bands common for all honeys, since it is known that some of the enzymes found in honey are provided by the bees (Bauer et al., (1996). Proteins such as glucose oxidase,  $\alpha$  and  $\beta$  glucosidase and amylase are regular components in honeys Simuth et al., (2004).

The protein profile of tested honeys differed in other polypeptide bands which probably derived from pollen and/or nectar. The polypeptide bands of 38 kDa and 25kDa observed in this study have been reported to occur in unifloral honeys (sunflower honey). These protein bands in honey used in this study were present in low concentrations as judged from the intensity of their staining in gels. This is in agreement with literature data that the amount of the common honeybee proteins are much higher than those originated from nectar or pollen (Baroni et al., 2002).



New Zealand manuka honey (*Leptospermum scoparium*), used as a reference honey in my project, showed a protein profile different from the rest of the Canadian honeys, but possessed the polypeptides bands of 72, 55, 43 and 25kDa present in Canadian honeys.

A variety of proteins were found in honeys by other researchers, and their presence has been shown to be a useful indicator of the geographical and floral origins of honeys (Wong et al., 2009). Won et al., (2007) detected up to nineteen protein bands in honeys from different botanical origin in SDS gels using silver staining. Many of these proteins were of pollen origin. While some authors stated that protein profiles in honey may be useful in assessing the floral origin of honeys, Gomes et al., (2009), Baroni et al., (2002). Baroni et al., (2002) reported that sometimes the concentration of pollen proteins in honey is below detection limit of the SDS-PAGE technique.

Similarly in my research, differences in protein profile in honeys from different botanical origin were observed. In SDS-PAGE, conducted under reducing conditions, dark honeys originating from buckwheat exhibited a "long pattern" of polypeptides that consisted of 8 bands. The bands were of high intensity after silver staining. In contrast, light honeys such as 65 and 66 possessed a "short" pattern consisting of 1 to 4 bands and the bands were of low intensity.

In summary, the protein profiles and intensity of protein bands in SDS-gels showed variability in honeys of different botanical origin, with buckwheat honeys possessing the highest number of polypeptides.

### ***Concentration of protein in honeys***

The protein concentration was established using the Pierce Protein Assay. The values of protein concentration in the tested honeys ranged from 96.2 to 3344.6ug/ml. Light color honeys (wild flower H179) showed the lowest protein content while the darker (buckwheat honey) H125 showed the highest. This data supports my results

obtained from SDS–PAGE, where darker honeys such as buckwheat honey showed higher number of protein polypeptides and a higher intensity than lighter color honeys.

The comparison of the protein concentration obtained in honeys used in this project with literature data is complicated by differences in methods used and differences in preparation of protein samples. In addition, honeys from different botanical sources were expected to possess varying amount of proteins. However, the range of protein concentrations in Canadian honeys was, in general terms, in the limits of those reported by other researchers. Wang et al., (2009) assessed that the protein content in honeys originated from Hawaii ranged from 496.3 to 688.9µg/ml. Gheldof et al., (2002) found that protein content of dark buckwheat honeys ranged from 1185.2 to 4088.9µg/ml.

The protein concentrations of Canadian honeys used in this study varied among honeys originating from different botanical sources and it was in the range with those reported in literature.

#### ***Instability of proteins in stored honeys***

Unexpectedly, the storage of honey at room temperature caused the disappearance of some protein bands in SDS–PAGE gels. In stored honeys, a change in the intensity and sharpness of bands was observed. The disappearance of proteins did not seem to be the result of a proteolytic degradation, since the incubation of bovine serum albumin (BSA) of known concentration with honeys did not produce albumin degradation products when incubation products were analyzed on SDS–PAGE gels.

Since undiluted honey is a supersaturated carbohydrate solution of high osmolarity and low water content, it could be argued that these conditions could not be supportive to any enzymatic activity. However Iglesias et al., (2006) reported the presence of protease and/or peptidase activity during the first few months of storage.

The disappearance of protein bands and appearance of diffused bands in SDS-gels have been observed during storage of wines and also in stored green coffee beans (Montavon et al., 2003a).

It has been reported that in the stored green coffee beans, the 11S storage protein formed adducts with polyphenols (Montavon et al., 2003b).

According to Montavon et al., (2003c), the disappearance of protein bands was a result of protein sequestration by polyphenols in green coffee beans. In the case of honeys, it has been suggested that proteins play a role in the deactivation of phenolics which are toxic to bees and bee larvae. The proposed mechanism included the oxidation of phenolic with hydrogen peroxide produced in honey and the binding of oxidized phenolics to proteins (Liu et al., 2005).

Considering the above literature data, we came to an assumption that the drastic decrease in the band intensity and sharpness observed in SDS-PAGE gels could be a result of proteins modification by phenolics. Next, the presence of phenolics in protein samples was simply demonstrated by the reaction with ferric chloride. The honey protein fraction, obtained by precipitation with 80% ethanol, formed a yellowish-brown color in reaction with ferric chloride indicating the presence of phenolic groups. In the following experiments, the presence of phenolics and their concentration in honey samples was precisely determined using the Folin-Ciocalteu method and is described in details below.

The results of my study demonstrated that honey protein fractions contained polyphenols. The disappearance of proteins in stored honey was possibly associated with complexation of proteins with phenolics.

### ***Protein-phenolic complexes***

Protein-polyphenol interactions, such as the interaction between proline -rich saliva proteins and food polyphenols, tanning of leather, or interactions of storage

proteins in seeds and polyphenols, are well described in literature. The protein–polyphenols interactions are also well known in the beverage industry. They are responsible for a formation of haze and colloids during production of wines and beers. Therefore, it was suspected that in honey, which is enriched in polyphenols, similar interactions with proteins could occur.

Some authors have suggested that non-covalent binding is the main kind of interactions between proteins and phenols and they have proposed some models. Jobstl et al., (2004) proposed a protein–polyphenol binding model that involves three stages. The first stage involves compaction of the protein upon binding to polyphenols, second stage involves addition of a second polyphenol coat to the compacted proteins and third stage involves a crosslinking of polyphenol-coated proteins and formation aggregates and large complexes.

Rawel et al., (2005) centered their attention to the role of non covalent interactions between proteins and polyphenols, although both covalent and non covalent binding are likely to take place simultaneously, as shown recently for the binding of chlorogenic acid to proteins in coffee beans.

### ***Removal of polyphenols from protein–polyphenol complexes***

To remove polyphenols from the proteins–polyphenol complexes, two methods of liquid extractions of honeys were tested; extraction with 80% ethanol (called protocol 1) and extraction with a mixture of three alcohols (ethanol, methanol and isopropanol, protocol 2). The efficiency of extraction with these two methods was determined based on the protein content in precipitates (Pierce protein assay), phenolic content in protein precipitates and supernatants (Folin–Ciocalteu method) and protein profiles in SDS–PAGE. Results of phenolic extractions using both methods were very similar. However since applying protocol 1 resulted in a loss of a protein band in SDS–PAGE, protocol 2 was the method of choice to continue working.

***Effect of storage time and temperature on the protein and phenolics concentrations in honey***

It is known that honey stored for prolonged periods of time loses some quality such as color, aroma, as well as some biological functions, such as antibacterial and antioxidant activity. The reason for deterioration of honey quality with time has not been sufficiently investigated.

Honey stored at different temperatures (Room temperature, 4 °C and -20 °C) all showed the reduction in protein concentrations measured by Pierce proteins assay. The drastic reduction in protein concentration was dependent on the temperature of storage; it was the highest in honeys stored at room temperature and lowest in honey stored at -20 °C.

There was no change in the position of polypeptide bands in the protein profile for the same honey during storage, however there was change in the concentration of polypeptides bands. The loss of proteins was more notable in dark buckwheat honeys than in light honeys and amounted to 42.5% and 41.8% respectively. Dark color buckwheat honey (H177) contained the highest amount of protein, 1465µg/ml. This amount was reduced to 623µg/ml after 4 months of storage. Light color honeys (H178 and H179) showed a lower protein concentration, 212 and 184µg/ml, respectively.

The rate of reduction in the honey protein content was the fastest during the first three months of storage and after this period a plateau was reached and the concentration of the protein remained more stable. According to the literature, there might be several reasons for protein reduction during storage. One of the reasons could be a potential enzymatic degradation of proteins in honey. In contrast to our findings, Iglesias et al., (2006) reported the presence of protease and/or peptidase activity during the first few months of storage.

One more reason for the protein loss during the storage time is the Maillard reaction that occurs in food with high sugar and amino acid/ protein content. In honeys, the presence of Amadori's compounds derived from the amino acids lysine, proline,  $\gamma$  aminobutyric acid, and arginine and glucose have been detected (Sanz et al., (2003). The presence of the advanced stage products of the Maillard reaction, melanoidins, has been recently found in honeys (Brudzynski and Miotto, 2010).

Initial differences in the concentration of proteins may not be related only to the botanical origin of honeys (Won et al., (2007). Each protein and enzyme in honeys has a biological purpose; it plays a role in the biochemical processing of honey constituents such as sugars. Simuth et al., (2009) called these proteins "technological enzymes". Apparently honeybees secrete and deposit the amount of protein that is needed for these processes. Von der Ohe, (1994) noted that the amount of proteins in honeys, which originate from the secretion of bees, is affected by the season the bees are working on the gathered nectar, the age of worker bees and on an abundance of blooming flowers.

It has been reported that some hypopharyngeal gland proteins of honey bee workers play an important role in deactivating phenolics in nectar, during the process of honey harvesting and it has also been reported that controlled nest homeostasis of honey bees helps deactivate phenolics. In studies carried out inside of the hive and outside of the hive it was found that phenolics can form irreversibly complexes with herbivores's enzymes when sufficient  $O_2$  is present (Liu et al., 2005), Shi and Di 2000). This raises the possibility that glucose oxidase (GOX) may be inhibited by phenolics in nectar outside of the hive.

My results demonstrated that the concentration of proteins differed in honeys of different botanical origin and that the protein content was drastically reduced during the first three months of honey storage. These findings were supported by the results from

my SDS-PAGE which demonstrated that in stored honeys the reduction of the number of polypeptides was observed and the polypeptide bands show low intensity of staining.

In conclusion, storage of honey had a detrimental effect on the content of honey proteins. In this project, it was demonstrated for the first time that the variability in the number of protein bands in the same type of honey observed in SDS-PAGE was linked to the storage of honeys.

### ***Changes in polyphenol content during storage***

Dark color buckwheat honey (H177) contained the highest concentration of total phenolics, measured using the Folin- Ciocalteu method. Total phenolic content obtained for buckwheat honey H177 was 456mg/kg and this value is in the range with those obtained by Gheldof, Wang and Engeseth, (2002) for buckwheat honey. The light color honeys (H178 and H179) showed lower values 163 and 138.6µg/ml (respectively). This is in agreement with results presented by others that honeys with dark color have a higher amount of total phenolic compounds (Gheldof and Engeseth, 2002; Meda et al. 2005).

Storage of honeys resulted in decrease in phenolic content. However, the decrease was not so remarkably high as in the protein case. Three-months storage of honey resulted in 70 % loss of proteins, while the total phenolic content decreased only by 2 %.

These results indicated that proteins were more sensitive to the storage conditions than polyphenols.

### ***Separation and identification of phenolics involved in protein-polyphenols complexes using LC/MS.***

The presence of phenolics in protein precipitates after extraction of honeys with protocol 2 demonstrated that some residual polyphenols are more tightly bound to proteins and were not removed using a mixture of three alcohols. Acetone extraction

removed additional amount of phenolic compounds from protein precipitates. Our interest was focused on the chemical nature of phenolics which showed higher affinity to proteins. For that experiment, buckwheat honey H177 was used because this honey was enriched in proteins compared to lighter honeys, therefore, it was expected that larger amounts of phenolics will be involved in protein–polyphenol interaction.

The LC/MS, was the method of choice for the separation and identification of phenolics and other compounds which interacted with proteins.

The LC chromatogram (chromatogram A) showed a typical distribution of compounds of buckwheat honeys which agreed with those published by others (Biesega and Pyrzynska, 2009). Gheldof et al., (2002). The LC chromatogram A consisted of three major peaks: peak A eluting from at RT 1,5 to 4.0 min contained the most hydrophilic components, second peak B eluting at RT 10 to 14.5 min contained hydrophilic/hydrophobic components, and peak C eluting at RT 14.5 to 28 min contained hydrophobic compounds. This chromatographic profile was visibly changed when the supernatant obtained, after extraction of honey with protocol 2, was subjected to LC/MS. The main difference was a marked reduction of intensity of the peak B eluting at 10 to 14 min, while the intensities of peak A and C were unchanged. This indicated that the majority of compounds present in peak B were not removed using a mixture of three alcohols and were still bound to the protein precipitate. The residual compounds removed by extraction with three alcohols were called “free phenolics” to differentiate them from phenolics bound to the protein pellet. Chromatogram B represented the free phenolics, phenolics that were not attached to proteins in honey and were easily removed from the sample by applying protocol 2.

The treatment of the protein pellet (dissolved in water) with acetone removed the majority of compounds present in the peak B (chromatogram C). Chromatogram C represents the protein– bound phenolics, the phenolic compounds that could not be



removed with protocol 2. Comparison of the three chromatograms A, B and C indicate that a significant portion of total phenolics is bound to proteins (chromatogram C).

The total phenolic content measured in the samples subjected to the LC/MS using the Folin–Ciocalteu method indicated that acetone extraction removed 59.6% of phenolics initially present in honey. Therefore, more than 50% of phenolics were involved in protein–polyphenol complexes.

The identification of compounds in each fraction were not part of my thesis. However it was of interest to know at what main chemical groups they represented.

By comparison of retention times and mass ions of polyphenol standards to those obtained in chromatograms B and C, it was possible to tentatively identify flavonoids as main polyphenols involved in the protein–polyphenols interaction present in the acetone fraction. Taking into consideration the retention time and the mass spectra of flavonoid standards we were able to identify two major flavonoids in honeys, Pinobanksin  $m/z$  and Pinobanksin methylate. Apigenin, Kaemferol and Myricetin were also identified, as well as, one of the phenolic acids: a cinnamic acid.

It is well established that flavonoids in honey occur in a glycosylated form. The presence of a hexose unit was identified from the appearance of 162 mass units, which correspond to the loss of a hexose such as glucose. Ferreres et al., (2007) reported that in honey, flavonoids are usually found as conjugated with sugars by O–glycosidic bonds, mainly on the hydroxyl at 7 position of the aglycone nucleus, at 3 position (flavonols), on the 4' hydroxyl and less frequently on other positions. C–glycosyl flavones are also found, exhibiting C–C bonds that belong almost exclusively to positions 6 and/or 8.

To my knowledge, this is the first time that flavonoids are recognized as the main polyphenols involved in protein complexations in honey.

In conclusion, the main findings of this study include a recognition of the protein–polyphenol interaction and changes in protein concentration during storage due to an increasing formation of protein–polyphenol complexes.

It has been found in this study that glycosylated flavonoids seemed to be compounds which are preferentially involved in polyphenol–protein binding.

These observations lead to the next questions related to the importance of the polyphenol–protein interactions and the complex formation for nutritional and biological functions of honey. The relevance of protein–polyphenol complexes should be further examined.

## 5. References

- Al, M.L, Daniel, D, Moise, A Bobis, O, Laslo, L, Bogdanov, S., 2009. Physicochemical and bioactive properties of different floral origin honeys from Romania. *Food Chem.* 112: 863–867.
- Aljady A. M and Yusoff K. M., 2003. Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties. *Turk J. Med. Sci.* 33: 229–236.
- Akbulut M, Musa M and Coklar H., 2009. Evaluation of antioxidant activity, phenolic contents and some physicochemical properties of several pine honeys collected from Western Anatolia. *Int. J. Food Sci. Nutr.* 60(7): 577–589.
- Antony S. M, Rieck J. R, Dawson P. L, 2000. Effect of dry honey on oxidation in turkey breast meat. *Poult. Sci.* 79: 1846–1850.
- Arguezo -Ruiz T and Rodriguez-Navarro A, 1975. Microbiology of Ripening Honey. *Appl. Environ. Microbiol.* 30(6):893–896.
- Astwood K, Lee B, and Manley-Harris M., 1998. Oligosaccharides in New Zealand Honeydew Honey. *J. Agric. Food Chem.* 46(12):4958–4962.
- Auerswald L, Schneider P, Gade G., 1998. Utilization of substrates during tethered flight with and without lift generation in the African fruit beetle *Pachnoda sinuate* (Cetoniinae). *J. Exp. Biol.* 201:2333–2342.
- Alvarez M. E, Pennel R. I, Meijer P-J. M, Ishikawa A, Dixon R. A, Lamb C., 1998. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92:1–20.

Baker H. G and Baker I., 1975. Studies of nectar-constitution and pollinator-plant coevolution. In: Gilbert LE, Raven PH (eds) Coevolution of animals and plants: Symposium V, first international congress of systematic and evolutionary biology, Boulder Colorado, University of Texas. *Press Austin: 100-140.*

Baroni M. V, Chiabrando G.A, Costa C and Wunderlin D.A., 2002. Assessment of the Floral Origin of Honey by SDS-PAGE Immunoblot Techniques. *J. Agric. Food Chem. 50 (6), 1362-1367.*

Bauer Lauer, M D, Kohlich A, Hirschwehr R, Siemann U, Ebner Herwig and Scheiner O., 1996. Food Allergy to Honey: Pollen or bee products? Characterization of allergenic proteins in honey by means of immunoblotting. *J Allergy Clin. Immunol. 97:65-73.*

Bennick A., 2002. Interaction of Plant Polyphenols with Salivary Proteins. *Crit. Rev. Oral Biol. Med.13(2):184-196.*

Beretta G, Granata P, Ferrero M, Orioli M, Facino R. M., 2005. Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorometric assays and chemometrics. *Anal. Chem. Acta 533:185-191.*

Bi Y. M, Kenton P, Mur L, Darby R, Draper J., 1995. Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. *Plant J 8:235-245.*

Biesaga M and Pyrzysnka K., 2009. Liquid chromatography/tandem mass spectrometry studies of the phenolic compounds in honey. *J. Chromat. A. (38):6620-6626.*

Bonvehí J.S, Torrento M.T and Raich Muntané J., 2000. Invertase activity in fresh and processed honeys. *Sci. Food Agric. 80:507-512.*

Bogdanov S., 1997. Nature and origin of the antibacterial substances in honey. *Lebensm.-Wiss. und Technol.*, 30(7):748–753.

Bogdanov, S, Ruoff, K, Persano Oddo L., 2004. Physico-Chemical methods for the characterization of unifloral honeys. A review. *Apidology* 35: 4–17.

Bogdanov S., 2010. *Book of Honey. Honey Composition. Bee Product Science. Chapter 5.*

Boukraa L, Benbarek H, and Moussa A., 2008. Synergistic action of starch and honey against *Candida Albicans* in correlation with Diastase number. *Braz. J. Microbiol.* 39 (1) 40–43.

Brudzynski K and Miotto D., 2010. The relationship between the content of reaction-like products and bioactivity of Canadian Honeys. *Food Chem., in press.*

Carter C, Shafir S, Shafir S, Yehonatan L, Palmer R. G, and Thornburg R., 2006. A Novel role for proline in plant floral nectars. *Naturwissenschaften.* 93: 72–79.

Carter C, and Thornburg R., 2004. Tobacco nectarin V is a flavin-containing berberine bridge enzyme-like protein with glucose oxidase activity. *Plant Physiol.* 134:460–469.

Carter C, Graham R, and Thorburg R. W., 1999. Nectarin I is a novel soluble germin-like protein expressed in the nectar of *Nicotiana* sp. *Plant Mol. Biol.* 41:207–206.

Castro-Vazquez L et al., 2008. Influence of Storage Conditions on Chemical Composition and Sensory Properties of Citrus Honey. *J. Agric. Food Chem.* 56, 1999–2006.

Chamnongpol S, Willekens H, Moeder W, Langebartels C, Sandermann H, Van Montagu M, Inze D, Van Camp W., 1998. Defense activation and enhanced pathogen tolerance induced by H<sub>2</sub>O<sub>2</sub> in transgenic tobacco. *Proc. Natl. Acad. Sci. USA* 95:5818–5823.

Charlton A. J, Baxter N. J, Lokman Khan M, Moir A. J.G. Moir, Haslam E, Davis A. P and Williamson M. P., 2002. Polyphenol/ Peptide binding and precipitation. *J. Agric. Food Chem.* 50, 1593–1601.

Chmieslewska H. R and Szczesna T., 1995. Composition and properties of Polish buckwheat Honey. *Current advances in Buckwheat research.* 114: 783–799.

Codex Alimentarius. 2001. Codex standard 12 Revised Codex Standards for Honey. Standard and Standard methods. 11. (<http://www.codexalimentarius.net>).

Cotte J. I, Casabianca H, Giroud B, Albert M, Lheritier J, Grenier-Loustulot M. I., 2004. Characterization of honey amino acid profiles using high pressure liquid chromatography to control authenticity. *Anal. Bioanal. Chem.* 378, 1342–1350.

Crane E., 1990. *The traditional hive products: honey and beeswax. In bees and beekeeping. Sci. Practice and World Resources, Heinemann Newnes: Oxford, UK.* 388–451.

Deinzer M. L, Thomason P. A, Burgett D. M, Issacson D. L., 1977. Pirrolizidine alkaloids: their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.) *Sci.* 195:497–499.

Ecroyd C. E, Franich R. A, Kroese H. W, Steward D., 1995. Volatile constituents of *Dactylanthus taylorii* flower nectar in relation to flower pollination and browsing by animals. *Phytochemistry* 40:1387–1389.

Ferreres F, Andrade P, Gil M. I, Tomas Barberan F. A., 1996. Floral nectar phenolics as biochemical markers for the botanical origin of heather honey. *Zeit Lebensmitht Untersuch Forsch* 202:40–44.

- Ferreres F, Gil-Izquierdo A, Andrade P.B, Valentao P. and Tomas-Barberan F.A., 2007. *Characterization of C-glycosyl flavones O-glycosylated by liquid chromatography-tandem mass spectrometry. J. Chromat. A. 1161, 214-223.*
- Frankel S, Robison M. R and Berenbaum M. R., 1998. Antioxidant capacity and correlated characteristics of 14 unifloral honeys. *J. Apic. Res. 27-31.*
- Gheldof N, Engeseth N. J., 2002. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of vitro lipoprotein oxidation in human serum samples. *J. Agric. Food Chem. 50:3050-3055.*
- Gheldof N, Wang X-H and Engeseth, N. J., 2002. Identification and Quantification of Antioxidant Components of Honeys from Various Floral Sources. *J. Agric. Food Chem. 50, 5870-5877.*
- Gonzalez-Miret M. L, Terrab A, Hernandez D, Fernandez-Recamales M. A and Heredia F. J., 2005. Multivariate Correlation between Color and Mineral Composition of Honeys and their Botanical Origin. *J. Agric. Food Chem. 53, 2574-2580.*
- Gomez-Caravaca A.M, Gomez-Romero M, Arraez-Roman, Segura-Carretero and Fernandez-Gutierrez A., 2006. *Advances in the analysis of phenolic compounds in products derived from bees. J. Pharmac. Biom. Anal. 41: 1220-1234.*
- Gomez, S, Dias G.L, Moreira L., Rodrigues P and Estevinho L., 2009. Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. *Food and Chem. Toxicology. Published by Elsevier Ltd.*
- Griebel C, Hess G., 1940. The vitamin C content of flower nectar of certain Labiatae. *Zeit Untersuch Lebensmittt 79:168-171.*

- Guizelan, G, Guizelan, R., 1971. Dones concernat les elements minéraux dans quelques sortes de miel. *Proceedings of 23<sup>rd</sup> International Congress on apiculture; Moscow, Apimondia Publishing: Bucharest, Romania. 592–593.*
- Hagerman A. E and Butler L. G., 1981. The specificity of the proanthocyanidin–protein interaction. *J. Biol. Chem. 4494–4497.*
- Harborne J. B., 1988. Flavonoids in the environment structure activity relationships. *Prog. Clin. Biol. Res. 280:17–27.*
- Havesteen B. H., 2002. The Biochemistry and Medical significance of the flavonoids. *Pharmacol. Ther. Nov–Dec; 96 (2–3):67–202.*
- Hertog M. G, Hollman, P. C. H, Venema D. P., 1992. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *J. agric. Food Chem. 40,1591–1598.*
- Grassnigg N, Leonhard B, Crailsheim K., 2003. Free amino acids in the haemolymph of honey bee queens (*Apis mellifera* L). *Amino acids 24:205–212.*
- Huidobro J. F and Simal J., 1984. Determinacion del color y de la turbidez en la mieles. *Anal. Bromatol., 36(2), 225–245.*
- Jaganathan S. K and Mandal M., 2009. Antiproliferative Effects of Honeys and its Polyphenols: A review. *Journal of Biomedicine and Biotechnology. Article ID 830616.*
- Jobstl E, O'Connell J, Patrick J, Fairclough A. and Mike P. Williamson., 2004. Molecular Model for Astrigency produced by Polyphenol/Protein Interactions. *Biomacromolecules, 5, 942–949.*
- Kleppet K., 1966. The effect of hydrogen peroxide on glucose oxidase from *Aspergillus niger*. *Biochem. 5:139–143.*



Kubo T, Sasaki M, Nakamura J, Sasagawa H, Ohashi K, Takeuchi H and Natori S., 1996. Change in the expression of Hypopharyngeal-Gland Proteins of the worker Honeybees (*Apis mellifera* L.) with Age and/or Role. *J. Biochem.* 119, 291–295.

Kubota M, Tsuji M, Nishimoto M, Wongchawalit J, Okuyama M, Mori H, Matsuit, Surarit , Svasti J, Kimura A and Chiva S., 2004. Localization of  $\alpha$ -Glucosidases I, II and III in Organs of European Honeybees, *Apis mellifera* L., and the Origin of  $\alpha$ -Glucosidases in Honeys. *Biosci. Biotechnol. Biochem.* 68 (11), 2346–2352.

Kucharski R, Maleszka R, Hayward D. C, and Ball E. E., 1998. A royal jelly protein is expressed in a subset of Kenyon cells in the mushroom bodies of the honeybee brain. *Die Naturwissenschaften*, 85:343–346.

Majtan J, Kovacova E, Bilikova K and Simuth J., 2006. The immunostimulatory effect of the recombinant apalbumin 1-major honeybee royal jelly protein-on TNF $\alpha$  release. *International Immunopharmacology* 6, 269–278.

Malika N, Mohamed F and Chakib A. E., 2005. Antimicrobiological Activities of Natural Honey from Aromatic and Medicinal Plants o Antibio-resistant Strain of Bacteria. *International J. Agric. Biol.* 1560-8530/2-289-293.

Manchado-Sarni P, Canal-Bosch J-M, Mazerolles G and Cheynier V., 2008. Influence of the Glycosylation of Human Salivary Proline-Rich Proteins in Their Interactions with Condensed Tannins. *J. Agric. Food Chem.* 56(20): 9563–9569.

Marshall T, & William K. M., 1987. Electrophoresis of honey: Characterization of trace proteins from complex biological matrix by silver staining. *Anal. Biochem.* 167, 301–303.

Marshall T and Williams M. K., 1987. Electrophoresis of honey: Characterization of trace proteins from complex Biological Matrix by Silver Staining. *Anal. Biochem.* 167, 301–303.

Mckibben J, Engeseth N. J., 2002. Honey as a protective agent against lipid oxidation in ground turkey. *J. Agric. Food Chem.* 50, 592–595.

Meda A, Lamien C. E, Romito M, Millogo J, Nacoulma OG., 2005. Determination of total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chem.* 91:571–577.

Mehmet A, Mehmet M. O and Colar H., 2009. Evaluation of antioxidant activity, phenolic, mineral contents and some physicochemical properties of several pine honeys collected from Western Anatolia. *International J. Food Sci. Nutr.* 60(7): 577–589.

Merken H. M. and Beecher G.R., 2000. Measurements of Food Flavonoids by High-Performance Liquid Chromatography: A Review. *J. Agric. Food Chem.* 48(3).

Micheu S, Crailsheim K, Leonhard B., 2000. Importance of proline and other amino acids during honeybee flight (*Apis mellifera carnica* Pollmann). *Amino acids* 18:157–175.

Middleton Jr. E., 1996. Biological properties of plant flavonoids: an overview. *Int. J. Pharmacognos* 34, 344–348.

Montavon P., Mauron A-F & Duruz E., 2003. *Changes* in green coffee protein profiles during roasting. *J. Agric. Food Chem.* 51: 2335–2343.

Montavon, P., Duruz, E., Rumo, G., & Pratz, G., 2003. Evolution of green coffee protein profiles with maturation and relationship to coffee cup quality. *J. Agric. Food Chem.* 51, 2328–2334.

Molan, P. C., 1996. Honey for the treatment of infections. *Bee-Inf.* 3:6–7.

Morales V, Sanz L, Olano A, and Corzo N., 2006. Rapid Separation on Activated Charcoal of High Oligosaccharides in Honey. *Chromatographia*, 64: 233–238.

Musser R. O, Cipollini D. F, Hum–Musser S. M, Williams S. A, Brown J. K and Felton G. W., 2005. Evidence that the Caterpillar Salivary enzyme Glucose Oxidase Provides Herbivore Offense in Solenaceous Plants. *Archives of Insect Biochemistry and Physiology.* 58:128–137.

National Honey Board, 2007. Honeys and bees. <http://www.honey.com/>.

Naqvi S. M. S, Harper A, Carter C. J, Ren G, Guirgis A, York W. S and Thornburg R. W., 2005. Nectarin IV, a potent endoglucanase inhibitor secreted into the nectar of ornamental tobacco plants. Isolation, cloning and characterization. *Plant Physiol* 139: 1389–1400.

Naurato, N, Wong P, Lu Y, Wroblewski K, Bennik A, 1999. Interaction of tanning with human salivary histatin. *J. Agric. Food Chem.* 47, 2229–2234.

Nicholson S. W, and Human H. 2008. Bees get a head start on honey production. *Biology letters. Physiology. The Royal Society.* 4: 299–301.

Ohasi K, Natori S and Kubo T., 1999. Expression of amylase and glucose oxidase in the hypopharyngeal gland with and age dependent role change of the worker honeybee (*Apis mellifera* L.). *European J. Biochem.* 265 (1)127–133.

Persano Oddo L, Pulcini P, Piazza M. G., 1990. Diastase activity in some unifloral honeys. *Apidologic* 21, 7-24.

Persano Oddo L, Piazza M. G, Pulcini P., 1999. Invertase activity in honey. *Apidology* 1999, 30, 57-65.

Persano Oddo L and Piro R, 2004. Main European unifloral honeys: descriptive sheets. *Apidologie* 35 (special issue): 538-581.

Peumans W. J, Smeets K, VN Nerum K, Van Leuven F, Van Damme E. J, M., 1997. Lectin and alliinase are the predominant proteins in nectar from leek (*Allium porrum* L.) flowers. *Planta* 201:298-302.

Plessi M, Bertelli D, Rastelli G, Albasini A, Monzani A., 1998. Fruits of ribes, rubus, vaccinium and prenus genus. Metal content and genome. *Fresenius J. Anal. Chem.* 361, 353-354.

Laemmli U. K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 227 (5259):680-685.

Liu F, He J and Fu W., 2005. Highly controlled nest homeostasis of honey bees helps deactivate phenolics in nectar. *Naturwissenschaften.* 92:297-299.

Iglesias M. T, Martin-Alvarez J, Polo M. C, Lorenzo C, Gonzalez M and Pueyo E., 2006. Changes in the Free Amino Acid Contents of Honeys During Storage at Ambient temperature. *J. Agric. Food Chem.* 54: 9099-9104.

Rawell H. M, Meidtnr K and Kroll J., 2005. Binding of Selected Phenolic Compounds to Proteins. *Journal of Agricultural Food Chemistry.* 53: 4228-4235.

- Rawell H. M, Simone K. F, Meidtner K, Kroll J and Schweigert F J., 2006. Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.* 50, 705–713.
- Rice–Evans, C. A, Miller N. J and Paganga G., 1997. Antioxidant properties of phenolic compounds. *Trends Plant. Sci.* 2: 152–159.
- Robards K, Li X, Antolovich M, Boyd S., 1997. Characterization of citrus by chromatographic analysis of flavonoids. *J. Sci. Food Agric.* 75, 87–101.
- Rodriguez–Arce A. L and Diaz N., 1992. The stability of beta–carotene in mango nectar. *J Agric. Univ. PR.* 76:101–102.
- Romeis T., 1999. Rapid Avr9– and Cf–9–dependent activation of MAP kinases in tobacco cell cultures and leaves: Convergence of resistance gene, elicitor, wound, and salicylate responses. *Plant Cell* 11:273–287.
- Roshchina V. V and Roshchina V. D., 1993. The excretory function of higher plants. *Springer, Berlin Heidelberg New York.*
- Ruiz–Argueso T. Z and Rodriguez Navarro A., 1975. Microbiology of ripening honey. *Appl. Microbiol.* 893–896.
- Sancho M. T, Muniategui, S, Sanchez P, Huidrobo J. F, Simal J., 1991. *Mieles del Pais Vasco XI. Evaluation de los distintos tipos de cenizas y su alcalinidad en funcion de la conductividad electrica en mieles.* *Anal. Bromatol.* 43, 311–324.
- Sanchez M. P, Huidobro J. F, Muniategui M. I and Sancho M. T., 2001. Evolution of Invertase Activity in Honey over two years. *J. Agric. Food Chem.* 49, 416–422.

Sanz, M. L del Castillo, Corzo M. D, N and Olano, A., 2003. 2-furoylmethyl amino acids and hydroxymethylfurfural as indicators of honey quality. *J. Agric. Chem.* 51, 4278-4283.

Sarabana K. J and Mandal M., 2009. Antiproliferative Effects of Honey and of its Polyphenols: A review. *J. Biomed. Biotech.* ID 830616.

Seo S, Okamoto M, Seto H, Ishizuka K, Sano H, Ohashi Y., 1995. Tobacco MAP kinase: A possible mediator in wound signal transduction pathways. *Sci.* 270:1988-1992.

Schmitzova J, Klaudiny J, Albert S, Hanes J, Shroder W and Schrockengost V., 1998. A family of royal jelly proteins of the honeybee *Apis mellifera* L. *Cell Mol. Life Sci.* 54:1020-30.

Simuth J, Bilikova K, Kovacova E, Kuzmova Z and Schroder W., 2004. Immunological Approach to Detection of Adulteration in Honey: Physiologically Active Royal Jelly Protein Stimulating TNF- $\alpha$  Release is a Regular Component in Honey. *J. Agric. Food Chem.* 52:2154-2158.

Snowdown J. A and Cliver, D. O., 1996. Microorganisms in honey. *International J. Food Microbiol.* 31: 1-26.

Stobiecki M., 2000. Application of mass spectrometry for identification and structural studies of flavonoids glycosides. *Phytochemistry* 54: 237-256.

Thornburg R.W, Carter C, Powel A, Rizhsky L, Mittler R, Homer H T., 2003. A major function of the tobacco floral nectar is defense against microbial attack. *Plant Syst. Evol.* 238:211-218.

- Tomas-Barberan F. A, Martos I, Ferreres F, Radovic B. S and Anklaam E., 2001. "HPLC flavonoid profiles as markers for the botanical origin of European unifloral honeys". *J. Sci. Food Agric.* 81(5) 485–496.
- Tsao R and McCallum J., 2010. *Fruit and Vegetable Phytochemicals Chemistry, Nutritional Value and Stability. Chemistry of Flavonoids.* 131–154. Wiley-Blackwell.
- Yan, Q. and Bennick, A., 1995. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J.* 311: 341–347.
- Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J and Perez-Alvarez J. A., 2008. *Functional properties of Honey, Propolis and Royal Jelly. J. Food Sci.* 73 (9).
- Vogel S., 1969. Flowers offering fatty oil instead of nectar (*Abstract N 229*). In: *Abstracts of the papers presented at the XI International Botanical Congress, August 24–September 2, and the international Wood Chemistry Symposium, September 2–4, 1969 eatle, WA (USA)* 260.
- Von der ohe W, Dustmann J. H, Ohe von der K., 1991. Proline as a criterium of the "ripeness" of honey. *Dtsch. Lebensm. Rundsch.* 87, 383–386.
- Von der ohe W. 1994. *Unifloral honeys: chemical conversion and pollen Reduction. Grana* 33: 292–294. ISSN 0017–3134.
- Wang J, Kliks M, Qu W, Jun W, Shi G and Qing X. L., 2009. Rapid Determination of Geographical Origin of Honey Based on Protein Fingerprinting and Barcoding using MALDITOF MS. *J. Agric. Food Chem.* 57, (21).
- Weston R. J, Mitchell K. R and Alle K. L., 1999. Antibacterial phenolic components of New Zealand manuka honey. *Food Chem.* 64, 295–301.

White J, Subers, M. H, Schepartz A. I., 1963. The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origine in a honey glucose-oxidase system. *Biochimica et Biophysica. Acta* 73, 57-70.

White J. W and Kushnir I., 1966. *Enzyme Resolution in Starch Gel Electrophoresis. Anal. Biochem.* 16: 302-313.

White J. W., 1975. Physical characteristics of honeys. *In: Crane, E. (ed) Honey, A Comprehensive Survey, 207-39. Hienemann, London, UK.*

Won S. Lee D. C, Ko S. H, Kim J. W and Rhee H. I., 2008. Honey major protein characterization ad its application to adulteration detection. *Food Research International.* 07.014.

Wong C. M, Wong K. H and Chen D. C., 2008. Glucose oxidase: natural occurrence, function, properties and industrial applications. *Appl. Microbiol. Biotechnol.* 78:927-938.

Wu G, Shortt B. J, Lawrence E. F, Levine E. B, Fitzsimmons K. C, Shah D. M., 1995. Diastase resistance conferred by expression of a gene encoding H<sub>2</sub>O<sub>2</sub>-generating glucose oxidase in transgenic potato plants. *Plant Cell* 7: 1357-1368.

Yan Q and Bennick A., 1995. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J.* 311: 341-347.